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| 13. ABSTRACT (Maximum 200 Words)<br><br>Our long term goal of this proposal was to study the role of Elk-1 gene in the progression of breast cancer. Our studies on the expression of Elk-1 in sporadic breast cancer suggests that changes in the levels of expression of Elk-1 protein can lead to breast cancer. Elk-1 proteins were found to be involved in the estrogen induced signal transduction pathway. Constitutive expression of Elk-1 and ΔElk-1 proteins in rat fibroblasts and human breast cancer cells induces apoptosis. Similarly, high level expression of BRCA1 protein in mouse fibroblasts and human breast cancer cells induces apoptosis on serum withdrawal or calcium ionophore treatment. These results suggest that Elk-1 and BRCA1 genes play a critical role in the regulation of apoptosis of human breast cancer cells. We have found the BRCA1 protein to associate with E2F transcriptional factors, Cyclins, Cyclin dependent kinases, CBP and p53 protein suggesting a function for these proteins in cell cycle regulation, tumor suppression, transcription activation, etc. Breast cancers have a reduced ability to undergo cell death. Treatments aimed at increasing the apoptotic threshold by BRCA1 and Elk-1 gene therapy may have the potential to prevent the progression of these cancers. |   |  |   |                            |
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## **INTRODUCTION**

The ets-oncogene superfamily contains a large number of genes sharing a conserved region of 85 amino acid called the “ETS domain” and a majority of the ets-oncogene superfamily members, which we and others have previously isolated, cloned and characterized are involved in leukemias, lymphomas and solid tumors (1-8). One of the members Elk-1 (4) was shown to form a SRF (serum response factor) dependent ternary complex with the SRE (serum response element) similar to P62TCF (ternary complex factor) (9). Elk-1 was previously shown to be involved in the growth factor mediated signal transduction pathway involving activation of MAP kinase (mitogen activated protein kinase), leading to the transcriptional activation of c-fos proto oncogene both of which are activated in tumor cells. Since the fos regulatory function of Elk-1 is dependent on the presence of a growth factor regulated transcriptional activation domain whose activity is dependent on phosphorylation by MAP kinase (10-13) and JNK kinases (Jun amino terminal kinase) (14) *in vivo* both of which are activated in tumor cells, we speculated that Elk-1 proteins may be obligatory intermediates in the estrogen and growth factor mediated signal transduction pathway leading to the progression of breast cancer. We have tested this hypothesis by studying the levels of expression of Elk-1 protein in breast tumor samples as well as in several breast cancer derived tumor cell lines and compared expression with histologically normal breast tumor samples by western blot analysis using the Elk-1 polyclonal antibody to correlate expression

with stages of the disease. We observed high levels of expression of Elk-1 protein (~3-8 fold higher) in samples derived from invasive breast cancer than normal breast. These results suggested that Elk-1 proteins may be involved in the progression of breast cancer.

Breast cancers have been described in patients overtime to change from an estrogen dependent in initial stages of the disease to a hormonally independent tumor. We therefore studied the expression of Elk-1 in MCF-7 cells that have been made quiescent by depletion of steroids and growth factors and stimulated to enter the cell cycle after the addition of E2 by RT-PCR analysis. We found a rapid induction of Elk-1 and  $\Delta$ Elk-1 RNA following growth stimulation by E2 in MCF-7 cells, indicating the involvement of Elk-1 in the E2 induced signal transduction pathway in human breast cancer cells. Furthermore we observed no significant difference in the expression of Elk-1 protein on immunoprecipitation analysis of  $^{35}$ S methionine labeled MCF-7 cells exposed to estrogen for different time intervals. One explanation for this could be that MCF-7 cells have been maintained *in vitro* in tissue culture for a long time and hence may have drifted far from their origin *in vivo*, which could explain the results found in breast tumor samples. As suggested by one of the reviewers, MCF-7 cells are E2 responsive and non-metastatic, whereas MDA-MB-231 cells or CAL-51 cells are estrogen independent, highly invasive and metastatic. We therefore plan to compare the expression of Elk-1 in these cell lines which would indicate the role of Elk-1 in early i.e., E2 dependent and late (invasive) breast cancer cell growth.

Since steroid hormone estrogen and peptide growth factors stimulate cellular proliferation of human breast cancer cells by inducing tyrosine phosphorylation of src family protein kinases and expression of c-fos proto oncogene, we subjected purified recombinant Elk-1 protein to *in vitro* kinase assay using src kinase. Our results suggests Elk-1 protein to be a target for c-src kinase.

We have previously investigated and found that high level expression of Elk-1 or  $\Delta$ Elk-1 protein transforms mouse fibroblasts *in vitro* and induces tumors in nude mice indicating that Elk-1 and  $\Delta$ Elk-1 proteins are potentially tumorigenic. These results correlated well with the results obtained using breast tumor samples. One should bear in mind that breast cells are epithelial in origin unlike mouse/rat fibroblasts which are mesenchymal.

Recently a familial breast and ovarian cancer susceptibility gene BRCA1 was identified (15) and shown to be either lost or mutated in families with breast and ovarian cancer (16-20). Earlier, results on the expression of BRCA1 mRNA in sporadic breast cancer indicated decrease in the level of BRCA1 mRNA levels during the transition from carcinoma in situ to invasive cancer (21). But recent results of us and others have indicated increase in the expression of BRCA1 in tumor compared to normal samples, similar to Elk-1 protein and BRCA1 expression has been correlated with the proliferation state of the cell (27, 28). At this juncture, we were tempted to look for any relation between BRCA1 expression and Elk-

1 expression or vice versa. So, in order to understand the role of BRCA1 in cell transformation, we obtained stable NIH3T3 cell lines expressing BRCA1 antisense RNA. The antisense BRCA1 expressing NIH3T3 cells showed accelerated growth rate, anchorage independent growth and tumorigenicity in nude mice unlike the parental transfectants (25, reprint enclosed). These results provide direct biological evidence for the possible function of BRCA1 as a tumor suppressor gene. These results suggested that inhibition of expression of BRCA1 protein is sufficient to achieve transformation and BRCA1 functions as a growth regulator in normal cells. Similar results were obtained by others using BRCA1 antisense oligonucleotides in MCF-7 cells (21). Previously, we have performed the SRE TK CAT (Serum Response Element, Thymidine Kinase, Chloramphenicol acetyl transference reporter vector) functional assay for checking the levels of Elk-1 protein in cells that have been transfected with antisense RNA to BRCA1 (BRCA<sup>AS</sup>) that have been developed by us. We observed high levels of CAT activity which made us to speculate that BRCA1 could be a regulator of Elk-1 gene. But we know that there are many Elk-1 related proteins which would also respond to this reporter like SAP-1, SAP-2, Fli-1, etc. and hence, these results need to be confirmed by western blot analysis.

In order to study whether any relationship exists between BRCA1 and Elk-1 we have developed mouse fibroblasts cell lines and human breast cancer cell lines expressing BRCA1 (26, see enclosed reprint). We have found that serum deprivation or calcium ionophore

treatment of BRCA1<sup>S</sup> transfectants resulted in programmed cell death (26, see enclosed reprint). These results indicate that BRCA1 genes may play a critical role in the regulation of apoptosis of human breast cancer cells. We have examined the expression of Elk-1 protein in the MCF-7 BRCA1<sup>S</sup> cells by western blot analysis and have observed higher levels of expression of Elk-1 compared to vector transfected MCF-7 cells. These results suggested that Elk-1 could be a target for BRCA1. In order to test the hypothesis whether Elk-1 lies downstream of BRCA1 in the apoptotic pathway and to investigate the role of Elk-1 in apoptosis of breast cancer cells, we have developed rat fibroblast cell lines and human breast cancer cell lines expressing Elk-1 proteins. We have found that constitutive expression of Elk-1 and  $\Delta$ Elk-1 proteins induce apoptosis when cells are treated with calcium ionophore. These results suggest that Elk-1 proteins may be downstream targets and inducers of apoptosis in human breast cancer cells. Recent evidence suggests that hormone dependent tumors like breast and ovarian cancers have a decreased ability to undergo apoptosis. It may be possible that inability to undergo apoptosis in these cancers may be due to the decreased levels of functional Elk-1 and BRCA1 proteins. These experiments will give us a clue as to the possible regulation of Elk-1 function in breast cancer. Future experiments will be directed towards studying the role of Elk-1 in apoptosis/growth and tumor suppression. It may be possible that treatments that are aimed at increasing the apoptotic threshold by Elk-1/BRCA1 gene therapy may have the potential to prevent the progression of these malignancies.

**BODY**

**Task 1**                      **COMPLETED**

As mentioned earlier, in an attempt to link Elk-1 gene to breast cancer, which forms the foundation for the work proposed in this proposal and represents Task 1 of this grant proposal, we have isolated total RNA from ~20 different sporadic cases of breast cancer and various breast cancer derived cell lines that are in different stages of the disease. Of these samples some are sets of paired samples which contain both normal breast and breast cancer from the same patient, the rest are unpaired. All these samples have been evaluated histologically by frozen sectioning and H & E (Hematoxylin and Eosin) staining as normal or malignant. We subjected these total RNA (~20  $\mu$ g) samples to Northern blot analysis as described previously (4). The Northern blot was hybridized to Elk-1 specific probe. Equal amounts of RNA was loaded in all the lanes. The signal obtained on Northern blot was very weak and the results were inconclusive so we resorted to alternative strategies like reverse transcriptase PCR (RT-PCR) of total RNA samples using Elk-1 ATG and termination primer which can distinguish between Elk-1 and  $\Delta$ Elk-1 transcripts. We used human  $\beta$ -actin primer (Clontech) as internal controls. Once again, we could not detect clear Elk-1 and  $\Delta$ Elk-1 bands by RT-PCR.



Since the Elk-1 mRNA levels doesn't necessarily reflect differences in the level of Elk-1 protein, we studied the expression of Elk-1 at the protein level by Western blot analysis using the Elk-1 polyclonal antibody available with us. It is because of these negative results that we had to deviate to alternate strategies like Western blot for studying the expression of Elk-1 protein. We observed elevated levels of expression of Elk-1 protein ~3-8 fold higher in samples derived from invasive breast cancer than in normal breast. Thus, our results indicating alterations in the expression of Elk-1 during the progress from normal to invasive breast cancer has strengthened the objectives of this proposal. **WITH THESE RESULTS WE HAVE COMPLETED TASK 1 OF THIS PROPOSAL.**

**Task 2**                      **IN PROGRESS**

**Task 2 (a)**                      **COMPLETED**

To test the hypothesis whether Elk-1 protein are intermediates in the E2 mediated proliferation of breast cancer cells, we have studied the expression of Elk-1 in MCF-7 cells that have been made quiescent by depletion of steroids and growth factors and stimulated to enter the cell cycle by the addition of E2 for 60 minutes as described in the proposal followed by RNA isolation. Total RNA from both control untreated and E2 stimulated were subjected to double stranded cDNA synthesis, RT-PCR analysis and Southern blot using Elk-

1 ATG and internal primers which can distinguish between the two alternately spliced forms Elk-1 and  $\Delta$ Elk-1. The RT-PCR analysis was also repeated using human  $\beta$ -actin, 5' and 3' primers as internal controls. We find rapid induction of Elk-1 and  $\Delta$ Elk-1 RNA following growth stimulation by E2 of MCF-7 cells. These results indicate involvement of the Elk-1 protein in the E2 induced signal transduction pathway in human breast cancer cells. Similarly, we have also examined the expression of Elk-1 protein in MCF-7 cells that have been stimulated to proliferate in the presence of E2 for different periods and have not observed any increase in the levels of Elk-1 protein. **WITH THESE RESULTS WE HAVE COMPLETED TASK 2 (a).**

Task 2 (b)           **COMPLETED**

We have studied the autonomous and SRF dependent transcriptional activation of Elk-1 and  $\Delta$ Elk-1 proteins in MCF-7 cells that have been stimulated with E2 and growth factors. We have observed no difference in the transcriptional activation of Elk-1 proteins in presence of E2 and growth factors. **WITH THESE RESULTS WE HAVE COMPLETED TASK 2 (b).**

Task 2 (c)           **COMPLETED**

We have subjected purified recombinant Elk-1 protein to *in vitro* kinase assay, using purified c-src kinase. The Elk-1 protein was phosphorylated by c-src kinase, indicating that Elk-1 could be a physiological target of c-src kinase.

Task 2 (d)                    **COMPLETED**

We have studied the expression of Elk-1 proteins in MCF-7 cells (E2 responsive and non-metastatic) and CAL-51 cells (E2 independent, highly invasive and metastatic) by western blot analysis. We have observed higher levels of expression in CAL-51 cells compared to MCF-7 breast cancer cells. These results suggest a role for Elk-1 in late breast cancer cell growth.

Task 2 (e)                    **COMPLETED**

In an attempt to identify proteins that might interact with Elk-1 proteins, we have used an alternate methodology other than the yeast match maker two-hybrid system. Since Elk-1 is a physiological substrate for MAP kinase and has multiple MAPK phosphorylation sites, we purified and labeled the protein *in vitro* using MAP kinase and used it to screen a human breast tumor GT11 expression cDNA library. We have obtained several clones in the primary screening. We have not pursued these results any further due to insufficient funds.

Task (f) **COMPLETED**

Since we found elevated levels of expression of Elk-1 in breast tumor samples, we wanted to investigate whether high level expression of Elk-1 or  $\Delta$ Elk-1 proteins could lead to cell transformation. We have recently obtained stable BALB and Rat-1 cells expressing high levels of Elk-1 proteins based on immunoprecipitation, western analysis and functional assay. In these cells we have observed stimulation of transactivation of the fos promoter CAT reporter or SRE-TK CAT reporter plasmid unlike the control Rat-1 cells. We have used this as a functional assay for testing the presence of Elk-1 proteins. We find ~20 fold increase in reporter CAT activity after transfection of SRETKCAT reporter into Rat-1 Elk-1 cells when compared to parental Rat-1 cells. The Elk-1 and  $\Delta$ Elk-1 expressing Rat-1 cells showed anchorage independent growth, formed colonies in soft agar induced tumors in nude mice, unlike the control cells. These results suggests that Elk-1 gene has transforming properties at least in mesenchymal cells. **WITH THIS WE HAVE COMPLETED TASK 2 (f).**

Task 2 (g) **COMPLETED**

As mentioned previously, BRCA1 a familial breast and ovarian cancer susceptibility gene, was cloned and shown to be lost or mutated in families with breast and ovarian cancer

resulting in loss of function of the BRCA1 protein. Since the reported BRCA1 cDNA sequence is a composite of several partial cDNA sequences, we obtained a novel full length alternately spliced BRCA1 cDNA clone (33). Recently we have also cloned the variant BRCA2 cDNA (38) and have studied its function (39). To test the hypothesis whether BRCA1 functions as a tumor suppressor gene, we have used antisense RNA methodology (22, 23) and cloned the full length antisense BRCA1a cDNA into pcDNA3 expression vector (25). We have previously used this method to inhibit Ewing's sarcoma in mice (23, 24). We have transfected NIH3T3 cells with pcDNA expression vector containing antisense BRCA1 cDNA and obtained stable neo-resistant cell lines expressing antisense RNA to BRCA1. These cell lines showed ~5 fold decrease in the expression of endogenous BRCA1 protein by western blot analysis (25). The BRCA1<sup>AS</sup> cells showed accelerated growth rate, anchorage independent growth, tumorigenicity in nude mice unlike the parental cells (25). Transfection of SRE TK CAT reporter plasmid into BRCA1<sup>AS</sup> cells showed high levels of CAT activity unlike the parental NIH3T3 cells. These results provide the first direct biological evidence for the possible function of BRCA1 as a tumor suppressor. This high level of SRE TK CAT activity made us to speculate that BRCA1 could be a regulator of Elk-1 gene. But we know that there are many Elk-1 related proteins which respond to this reporter like SAP1, SAP2, etc. and hence these results have to be confirmed by western blot analysis. **WITH THESE RESULTS WE HAVE COMPLETED TASK (g).**

Task 2 (h)            **IN PROGRESS**

We plan to examine expression of Elk-1 proteins in mouse fibroblast cells that have been transfected with antisense BRCA1 by western blot analysis

Task 2 (i) & (j)       **COMPLETED**

Since our preliminary results suggested a link between Elk-1 and BRCA1 proteins, we studied the subcellular localization, phosphorylation and protein-protein interactions of BRCA1 proteins. Recently, we have characterized two new variant BRCA1 proteins (BRCA1a/p110 and BRCA1b/p100) which are phosphoproteins containing phosphotyrosine (33, reprint enclosed). Immunofluorescence and western blotting analysis indicated cytoplasmic and nuclear localization of BRCA1a and BRCA1b proteins (33, reprint enclosed). Recently, we have also shown the amino-terminal region of BRCA1a (BNT) but not BRCA1b can function as a transcriptional activator when fused to GAL4 DNA binding domain (34, reprint enclosed). Thus, BRCA1/1a proteins contain two autonomous transcriptional activation domains, one at the amino-terminal region (BNT) and the other at the carboxy-terminal region (BCT) (34). In an attempt to isolate proteins that interact with BRCA1, we have detected two cellular proteins (p65 BIP, p32 BIP) that specifically interact with BRCA1 (33, reprint enclosed). Western blot analysis of BIP indicated association with

E2F, cyclins, and CDK's and *in vitro* translated BRCA1a and BRCA1b proteins interacted directly with transcription factors E2F-1, E2F-4, cyclins A, B1, D1 and cyclin dependent kinases cdc2 and cdk2 suggesting a role for BRCA1 proteins in cell cycle regulation (33, reprint enclosed).

Recently, we have also found BRCA1a/1b proteins to interact both *in vitro* and *in vivo* with the carboxy-terminal domain of transcription factor CBP (35, reprint enclosed) as demonstrated by mammalian two-hybrid assays, co-immunoprecipitation/western blot studies, GST binding assays and histone acetyltransferase (HAT) assays of BRCA1 immunoprecipitates from human breast cancer cells (35, reprint enclosed).

Our recent results suggest BRCA1a and BRCA1b proteins function as co-activators of p53 tumor suppressor protein similar to BRCA1 (36, reprint enclosed). This study demonstrates for the first time the presence of a second p53 interaction domain in the carboxy-terminal BRCT domain of BRCA1 which is sufficient for activation of p53 dependent transactivation of the p21<sup>WAF1/CIP1</sup> promoter (36). The BRCA1a/1b proteins interact with p53 both *in vitro* and *in vivo*. The BRCT domain binds to the central domain of p53 which is required for sequence specific DNA binding (36). This BRCT domain also binds *in vitro* to CBP. These results suggest that one of the mechanisms by which BRCA1 proteins function is through recruitment of CBP/p300 associated HAT/FAT activity for acetylation of p53 to specific promoters resulting in transcriptional activation (36).

In order to study whether any relationship exists between BRCA1 and Elk-1 we have developed mouse fibroblasts cell lines and human breast cancer cell lines expressing BRCA1 (26, see enclosed reprint). We have found that serum deprivation or calcium ionophore treatment of BRCA1<sup>s</sup> transfectants resulted in programmed cell death (26, see enclosed reprint). These results suggested that Elk-1 could be a potential target for BRCA1. **WITH THIS WE HAVE COMPLETED TASK 2 (i) (j).**

Task 2 (k)

Previously we have observed high levels of expression of Elk-1 protein in MCF-7 cells that have been transfected with BRCA1 compared to vector transfected MCF-7 cells by western blot analysis using Elk-1 polyclonal antibody. These results suggested that BRCA1 gene may regulate the expression of Elk-1 or Elk-1 could be a downstream target for BRCA1 in the apoptotic pathway. Since BRCA1 is an inducer of apoptosis we speculated whether Elk-1 could also be involved in apoptosis. Recently, mitogen activated/extracellular response kinase kinase kinase (MEKK), a serine-threonine kinase that regulates phosphorylation and activation of mitogen-activated protein kinases (MAPK) was shown to induce cell death (29). Inducible expression of activated MEKK (which induces apoptosis) stimulated the



transactivation of c-Myc and Elk-1 (29). c-Myc and c-jun transcription factors which are regulated by MAPK phosphorylation also induce apoptosis (30). Since Elk-1 protein regulates c-Fos oncogene and is a target for MAPK and JNK both of which are activated by MEKK, we speculated whether it could play a similar role in inducing an apoptotic response. To test this hypothesis, we have transfected Rat-1 fibroblasts with pcDNA expression vector or pcDNA expression vectors containing human Elk-1 and  $\Delta$ Elk-1 cDNA and obtained stable G418 resistant cell lines expressing Elk-1 and  $\Delta$ Elk-1 proteins as described previously (26).

*Analysis of expression of Elk-1 and  $\Delta$ Elk-1 proteins in Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 transfectants*

We analyzed the expression of Elk-1 and  $\Delta$ Elk-1 proteins in Elk-1 and  $\Delta$ Elk-1 transfectants by immunoperoxidase staining (37, reprint enclosed), indirect immunofluorescence analysis (37) and western blot analysis (37) using Elk-1 polyclonal antibody as described previously (26 and 33). The nuclear and cytoplasmic staining was brighter and stronger in both Elk-1 and  $\Delta$ Elk-1 transfected cells compared to parental Rat-1 fibroblast cells suggesting higher level of expression of Elk-1 and  $\Delta$ Elk-1 proteins than Rat-1 cells. Western blot analysis revealed a significant increase in the levels of expression of Elk-1 and  $\Delta$ Elk-1 proteins when compared to the parental Rat-1 cells (37 ).

*Constitutive Elk-1 and  $\Delta$ Elk-1 expression induces cell death in Rat-1 cells after calcium ionophore treatment*

Previously, we have shown calcium ionophore A23187 to induce apoptosis in BRCA1a transfected NIH3T3 and MCF-7 cells (26). This led us to examine apoptosis in Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 transfectants after A23187 treatment. Rat-1, Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 were treated with calcium ionophore A23187 for 48 hours and the cell cycle distribution was determined by flow cytometry with propidium iodine staining method (31). Histogram of the DNA content and the percentage of cells in G1, S, and G2 plus M phase of the cell cycle were evaluated using EPICS profile analyzer. The Elk-1 and  $\Delta$ Elk-1 transfected cells showed accelerated rates of apoptosis (Ap value 49% for Rat-1 Elk-1 and 42% for Rat-1  $\Delta$ Elk-1 cells) in the presence of calcium ionophore (37) whereas the control Rat-1 fibroblasts cells showed lower levels of apoptosis under identical conditions (Ap value 16%).

*Apoptosis in the Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 cells confirmed by cell viability staining*

Measurement of apoptosis through the sub G1 peak in the DNA histogram gives no distribution between viable and dead cells since all the cells are fixed. We, therefore, studied the viability of Rat-1 cells, Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 cells cultured in the presence of calcium ionophore by crystal violet staining. Cell viability staining showed that most of the Elk-1 and  $\Delta$ Elk-1 cells treated with calcium ionophore A23187 were dead whereas most of

the control Rat-1 cells survived (37). These results suggest that constitutive expression of Elk-1 and  $\Delta$ Elk-1 proteins induce death in Rat-1 fibroblast cells.

*Apoptosis in the Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 cells confirmed by chromatin condensation*

Elk-1 and  $\Delta$ Elk-1 transfected Rat-1 cells were cultured in the presence of calcium ionophore to induce apoptosis and the incidence of cell death was determined by phase contrast microscopy after staining the cultures with Hoechst 33258 (32). Majority of the nuclei of Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 cells showed strong chromatin condensation and nuclear degradation into small, spherical nuclear particles of condensed chromatin characteristic of apoptosis. Whereas the parental Rat-1 cells did not show any significant change in the staining pattern (37).

*Apoptosis in the Elk-1 and  $\Delta$ Elk-1 transfectants confirmed by DNA fragmentation*

The induction of apoptosis in the Elk-1 and  $\Delta$ Elk-1 transfectants upon treatment with calcium ionophore was further confirmed by analysis of DNA fragmentation. The DNA of Elk-1 and  $\Delta$ Elk-1 cells treated with calcium ionophore was broken into oligo nucleosomal DNA ladder typical of apoptosis whereas the parental Rat-1 cells had no significant DNA degradation (37). These results suggested that calcium ionophore induces apoptosis more readily in Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 cells than Rat-1 cells. All the above results shown

for a single clone of Rat-1 Elk-1 or Rat-1  $\Delta$ Elk-1 cells have been reproducibly obtained with several other independent clonal isolates of Rat-1-Elk-1 and Rat-1- $\Delta$ Elk-1 cells. **WITH THESE RESULTS WE HAVE COMPLETED TASK 2 (k).**

### Task 3

#### Task 3(a) **COMPLETED**

In an attempt to study the role of Elk-1 gene in the regulation of apoptosis of human breast cancer cells, we have transfected MCF-7 cells with pcDNA expression vector or pcDNA expression vector containing human Elk-1 and  $\Delta$ Elk-1 cDNA's and obtained stable G418 resistant cell lines expressing Elk-1 and  $\Delta$ Elk-1 proteins.

#### *Morphology of MCF-7 Elk-1 and MCF-7 $\Delta$ Elk-1 transfectants*

The morphology of the Elk-1 transfectants were different than that of the parental Rat-1 fibroblast cells. The Elk-1 transfectants were slow growing and appeared to be flatter and larger compared to the parental MCF-7 cells. The morphology of the  $\Delta$ Elk-1 transfectants were similar to that of the parental MCF-7 cells (37). These MCF-7 Elk-1 and MCF-7  $\Delta$ Elk-1 cell lines were analyzed for Elk-1 and  $\Delta$ Elk-1 protein expression by immunoperoxidase staining and western blot analysis and found to express Elk-1 and  $\Delta$ Elk-1 proteins.

*Constitutive expression of Elk-1 and  $\Delta$ Elk-1 proteins induces apoptosis in MCF-7 cells treated with calcium ionophore*

Apoptosis in the MCF-7 Elk-1/ $\Delta$ Elk-1 transfectants were analyzed after treatment with calcium ionophore A23187. MCF-7 and MCF-7 Elk-1/ $\Delta$ Elk-1 cells were treated with calcium ionophore A23187 for 24 hours and the cell cycle distribution was analyzed by flow cytometry with propidium iodide staining. The Elk-1 transfected MCF-7 cells showed accelerated rates of apoptosis (Ap value 72%) in the presence of calcium ionophore (37), unlike the parental MCF-7 cells which showed very low levels of apoptosis (Ap value 4%). The MCF-7  $\Delta$ Elk-1 cells showed low levels of apoptosis in presence of calcium ionophore (Ap value 14%). The viability of MCF-7 cells, MCF-7 Elk-1 and MCF-7  $\Delta$ Elk-1 cells cultured in the presence of calcium ionophore were tested by crystal violet staining. Cell viability staining showed that a vast majority of the MCF-7 Elk-1 cells treated with calcium ionophore A23187 were dead whereas most of the control MCF-7 cells survived. These results suggest that Elk-1 induces death in MCF-7 cells. The induction of apoptosis in the MCF-7 Elk-1 and MCF-7  $\Delta$ Elk-1 transfectants was further confirmed by DNA fragmentation analysis upon treatment with calcium ionophore. The DNA of MCF-7 Elk-1 cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder unlike the parental MCF-7 cells (37). The DNA fragmentation observed in MCF-7  $\Delta$ Elk-1 cells was much less. These results suggest that overexpression of Elk-1 in breast cancer cells accelerates

apoptosis. We plan to identify the role of DNA binding, transcriptional activation and protein protein interactions on the apoptosis inducing function of Elk-1 proteins in breast cancer cells.

Task 3 (b) (c) (d) **IN PROGRESS**

We have introduced Elk-1 into E2 independent, highly invasive and metastatic breast cancer cell line CAL-51. We plan to study the effect on apoptosis and growth/tumor suppression. We also plan to study whether DNA binding, transcriptional activation and protein-protein interactions play a role in the apoptosis inducing, growth/tumor suppressor function of Elk-1 proteins. We have not been able to complete all the proposed work in the grant proposal due to insufficient funds available in the funded grant proposal. In fact, I had written a letter asking for additional funds for a year so that I could finish the work proposed but, unfortunately, this request was turned down by the U.S. Army Medical Research and Materiel Command.

## **CONCLUSION**

Our studies are designed to investigate the role of Elk-1 in the progression of breast cancer. In summary, our results suggest that BRCA1, which is a tumor suppressor and Elk-1 gene product function as inducers of apoptosis in breast cancer cells. This study demonstrates for the first time a role for Elk-1 proteins in mediating apoptosis.

Recent evidence suggests that hormone-dependent tumors like breast and ovarian cancers have a decreased ability to undergo apoptosis. Our results suggest that lack or decreased levels of expression of functional BRCA1 or Elk-1 gene products in breast cancer may be responsible for the increased resistance of these cells to undergo apoptosis. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 or Elk-1 gene therapy may have the potential to prevent the progression of these malignancies. Alternatively, one can use therapeutic agents that can activate BRCA1/Elk-1 downstream signals involved in apoptosis for the treatment of breast cancers. Results from this work would be utilized in the future for early detection, diagnosis and also treatment of breast cancer.

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**MEETING ABSTRACTS**

1. Shao, N., Chai, Y.L., Reddy, E.S.P. and Rao, V.N. "BRCA1 induces apoptosis of human breast cancer cells." Presented at the International Meeting on programmed cell death at Shanghai, China, September, 1996.
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3. Chai, Y.L., Shao, N., Chipitsyna, G., Reddy, E.S.P. and Rao, V.N. "BRCA1 Splice Variants, Phosphorylation, Tumor Suppression, Apoptosis and Protein-Protein Interaction" Presented at the Drexel and MCP Hahnemann Sigma Xi Research Day, May 6, 1999.
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5. Chai, Y.L., Shao, N., Chipitsyna, G., Reddy, E.S.P. and Rao, V.N. BRCA1 Splice Variants, Phosphorylation, Tumor Suppression, Apoptosis and Protein-Protein Interaction. Presented at the Pan-Pacific Lymphoma Conference, July, 1999.



**Veena N. Rao, Ph.D.**

**PERSONNEL**

1. Ningsheng Shao
2. Yu Li Chai

# The EWS gene, involved in Ewing family of tumors, malignant melanoma of soft parts and desmoplastic small round cell tumors, codes for an RNA binding protein with novel regulatory domains

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The EWS gene, which maps to band q12 of human chromosome 22, is involved in a wide variety of human solid tumors including Ewing sarcoma, related primitive neuroectodermal tumors, malignant melanoma of soft parts and desmoplastic small round cell tumors. In these tumors, the EWS is fused to genes encoding transcriptional activators/repressors, like Fli-1 or *erg* or ATF 1 or *wt1*. To better understand the function of the EWS protein, we cloned the EWS cDNA. Sequence analysis of this cDNA revealed differential splicing involving two exons encoding 72 amino acids. Both alternatively spliced transcripts, EWS and EWS-b, are expressed in a variety of cells. Because EWS proteins contain putative conserved RNA binding motifs, we studied the RNA binding properties of the EWS protein. The EWS-b protein binds to RNA *in vitro* and, specifically, to poly G and poly U. The RNA binding activity was localized to the carboxy terminal 86 amino acids, which constitute RGG box. Thus the amino terminal domain of EWS (NTD-EWS), which is involved in chromosome translocation may regulate the specificity of RNA binding activity of EWS. An EWS-*erg* chimeric protein, which is found in Ewing's sarcoma cells, functions as a transcriptional activator. Mutational analysis of EWS-*erg* chimeric protein revealed that NTD-EWS functions as a regulatory domain for the transcriptional activation properties of EWS-*erg* chimeric protein.

## Introduction

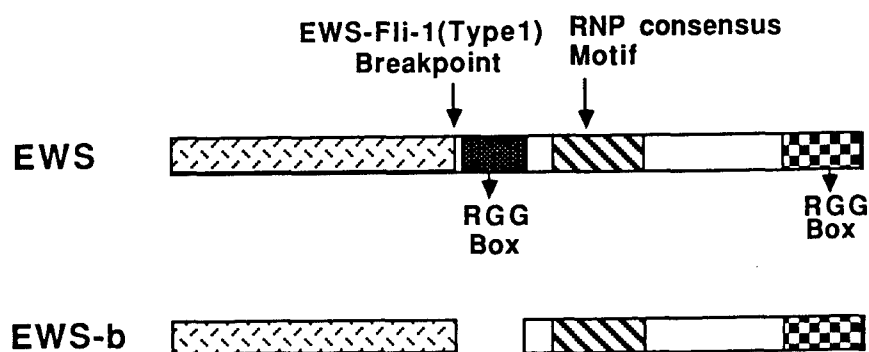
Chromosome abnormalities are responsible for haematological neoplasias. In leukemias, chromosomal translocations result in the activation of oncogenes or fusion of two or more genes. Although the incidence of solid tumors is much higher compared with leukemias, little progress has been made in the molecular analysis of solid tumors. Recent studies indicate human solid tumors contain recurrent chromosome translocations, suggesting that, as with leukemias, these chromosome abnormalities may be responsible for the human malignancy.

Two closely related human solid tumors, Ewing's sarcoma and peripheral neuroepithelioma carry either t(11; 22)(q24;q12) or t(21; 22)(q22;q12) chromosome

translocations (Delattre *et al.*, 1992). These translocations produced hybrid genes between EWS, on chromosome 22, with either Fli-1 or *erg* genes, located on chromosomes 11 and 21, respectively. The *erg* and Fli-1 are members of the *ets* oncogene super family, which includes *c-ets-1* (a cellular homologue of *v-ets* gene) (Reddy and Rao, 1988; Coquilland *et al.*, 1988; Watson *et al.*, 1988; Chen, 1988), *ets-2* (Watson *et al.*, 1988; Bouloukos *et al.*, 1988), *erg* (Rao *et al.*, 1987; Reddy *et al.*, 1987), *elk-1* and *elk-2* (Rao *et al.*, 1989; Rao & Reddy, 1993), Pu.1/Spi-1 (Goebl *et al.*, 1990; Klemsz *et al.*, 1990), E74 (Burtis *et al.*, 1990), Fli-1 (Ben-David *et al.*, 1991; Prasad *et al.*, 1992; Watson *et al.*, 1992), GABP  $\alpha$  (LaMarco *et al.*, 1991), Sap-1 and Sap-2 (Dalton & Treisman, 1992), elf-1 (Thompson *et al.*, 1992), PEA3 (Xin *et al.*, 1992), *yan/pok* (Lai and Rubin, 1992; Tei *et al.*, 1992), ER 81 and ER 71 (Brown & McKnight 1992); Spi-1b (Ray *et al.*, 1992), ERP (Lopez *et al.*, 1994) *et al.*, 1994), *tel* (Golub *et al.*, 1994), D-elg (Pribyl *et al.*, 1988), PE-1 (Klemsz *et al.*, 1994), ERM (Monte *et al.*, 1994) etc. The majority of these *ets* proteins bind to DNA (Bouloukos *et al.*, 1990; Rao *et al.*, 1989; Reddy & Rao, 1990) and lead to sequence specific transcriptional activation (Wasylyk *et al.*, 1990; Gunther *et al.*, 1990; Bosselut *et al.*, 1990; Ho *et al.*, 1990; Klemsz *et al.*, 1990; Reddy & Rao, 1991; Rao & Reddy, 1992a; Urness & Thummel, 1990; Thompson *et al.*, 1992; Xin *et al.*, 1992; Ray *et al.*, 1992; Brown & McKnight, 1992; Rao *et al.*, 1993). Some of the *ets* proteins bind to DNA either autonomously or through association with other nuclear factors (Hipskind *et al.*, 1991; Dalton & Treisman, 1992; Rao & Reddy, 1992b; Brown & McKnight 1992; Pongubala *et al.*, 1993; Gegonne, *et al.*, 1993). In addition, the *ets* protein cooperates with other nuclear oncogenes in transcriptional activation (Wasylyk *et al.*, 1990; Dudek *et al.*, 1992).

Molecular characterization of another solid tumor, malignant melanoma of soft parts or soft tissue clear cell sarcoma, which shares t(12;22)(q13;q12), reveals fusion of EWS with another transcriptional factor gene, ATF1 (Zucman *et al.*, 1993). Similarly, the EWS gene is fused to the Wilm's tumor gene (*wt1*) in desmoplastic small round cell tumors (DSRCT) which show recurrent translocation, t(11;22)(p13;q12) (Ladanyi & Gerald, 1994). Thus, translocations involving the EWS gene and different genes encoding transcription factors are found in a variety of human solid tumors.

To assess the contribution of the EWS protein to the formation of these human solid tumors, it is necessary to know the normal function of the EWS protein. We cloned and sequenced the EWS cDNA and characterized it by nucleotide sequence analysis. The putative EWS protein



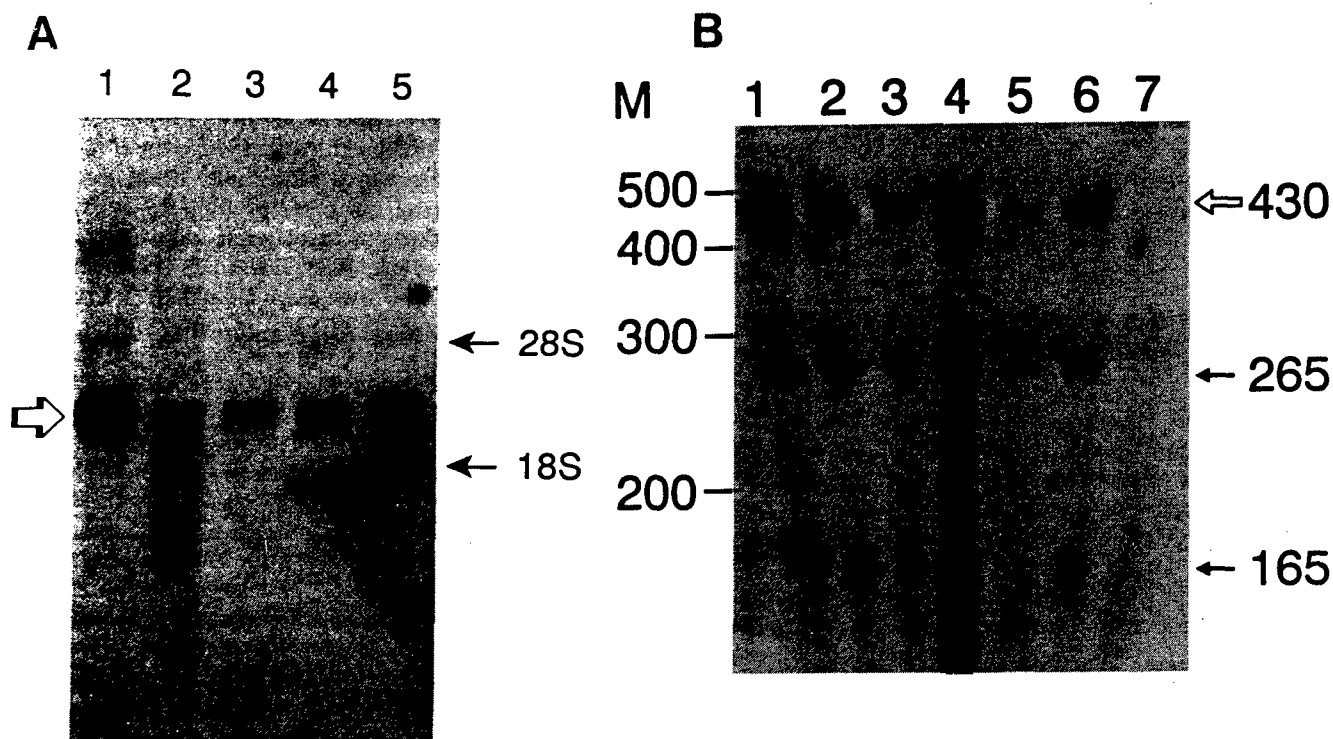
**Figure 1** Structure of the coding regions of the two EWS cDNAs representing EWS and EWSb. The region (exon 8 and 9) (Plougastel *et al.*, 1989) deleted as a result of alternative splicing is shown.

contained a conserved 85 amino-acid domain [termed the RNA recognition motif (RPM) or RNP-CS or RNA binding domain (RNA BD) or RNP motif or RRM] (Dreyfuss *et al.*, 1988; Kenan *et al.*, 1991; Haynes, 1992), which is found in several RNA-binding proteins. Therefore, the EWS protein may encode for RNA binding protein. In this report we show that alternative splicing produces two EWS transcripts, both of which encode RNA binding proteins. This binding is mediated and/or regulated by three domains. We also demonstrate that the EWS domain in an EWS-erg chimeric protein functions as a regulatory domain and regulates its transcriptional activation properties.

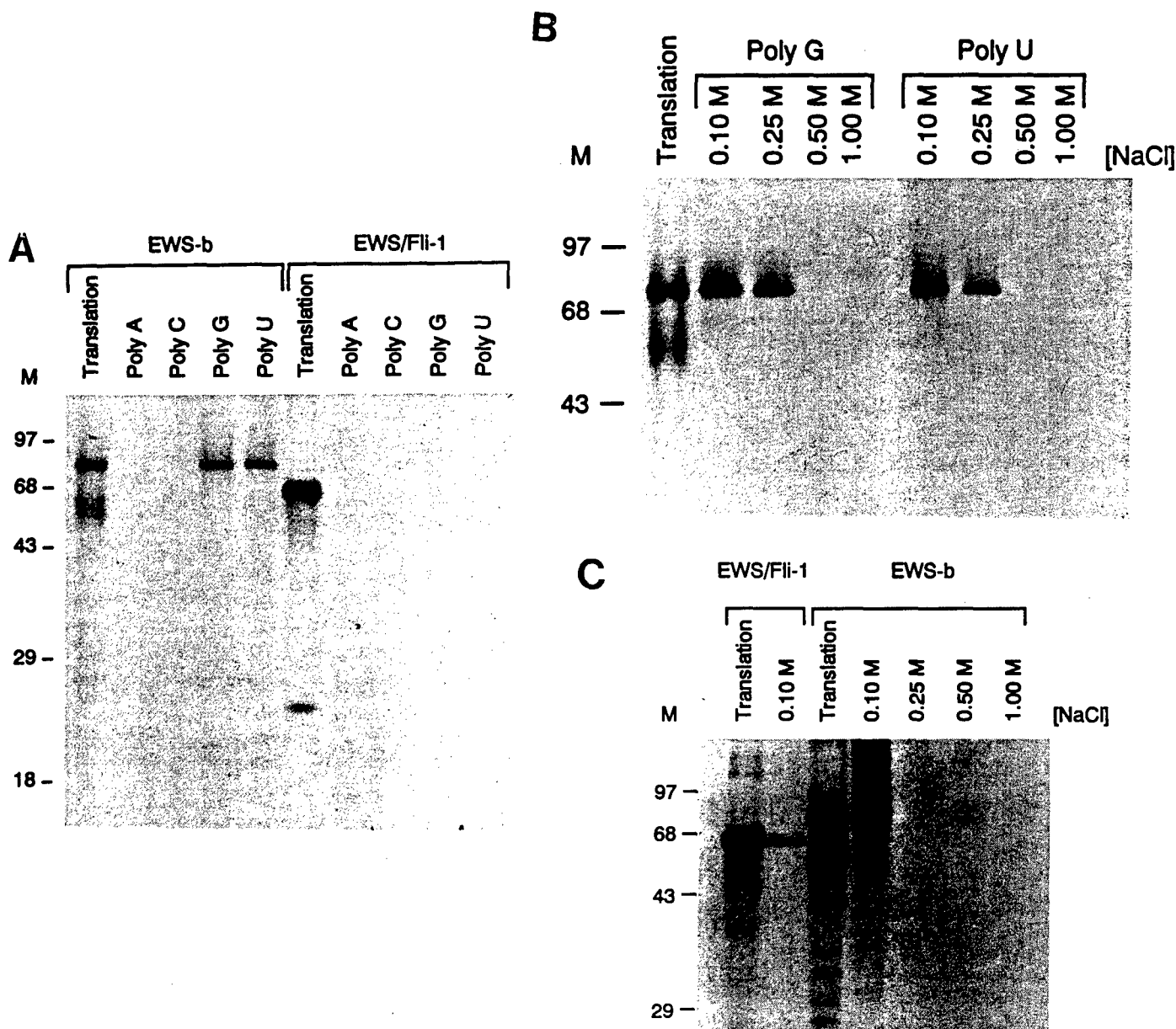
## Results and Discussion

### *Molecular cloning of EWS cDNA representing the variant EWS protein (EWS-b)*

In order to study the function of the EWS protein, we isolated a EWS cDNA by reverse transcription-PCR using RNA from Molt 4 cells. Sequence analysis of this cDNA showed that this cDNA represents an alternatively spliced transcript (EWS-b) of the EWS gene, with a deletion of two exons (exon 8 and 9) (Zucman *et al.*, 1993; Plougastel, *et al.*, 1993). The EWS-b transcript encodes a variant EWS polypeptide (Figure. 1) of 583 amino acids with an



**Figure 2** (a) Expression of EWS gene in a variety of human cells: Lane 1, Colo 320; lane 2, 697; lane 3, KG-1; lane 4, KG-1a; lane 5, Molt 4. (b) Analysis of the expression of EWS transcripts by RNAase protection assay. RNAase protection was carried out as described (Prasad *et al.*, 1994). Lane 1, Colo 320; lane 2, 697; lane 3, KG-1; lane 4, Molt 4; lane 5, HeLa; lane 6, T98G; lane 7, tRNA.

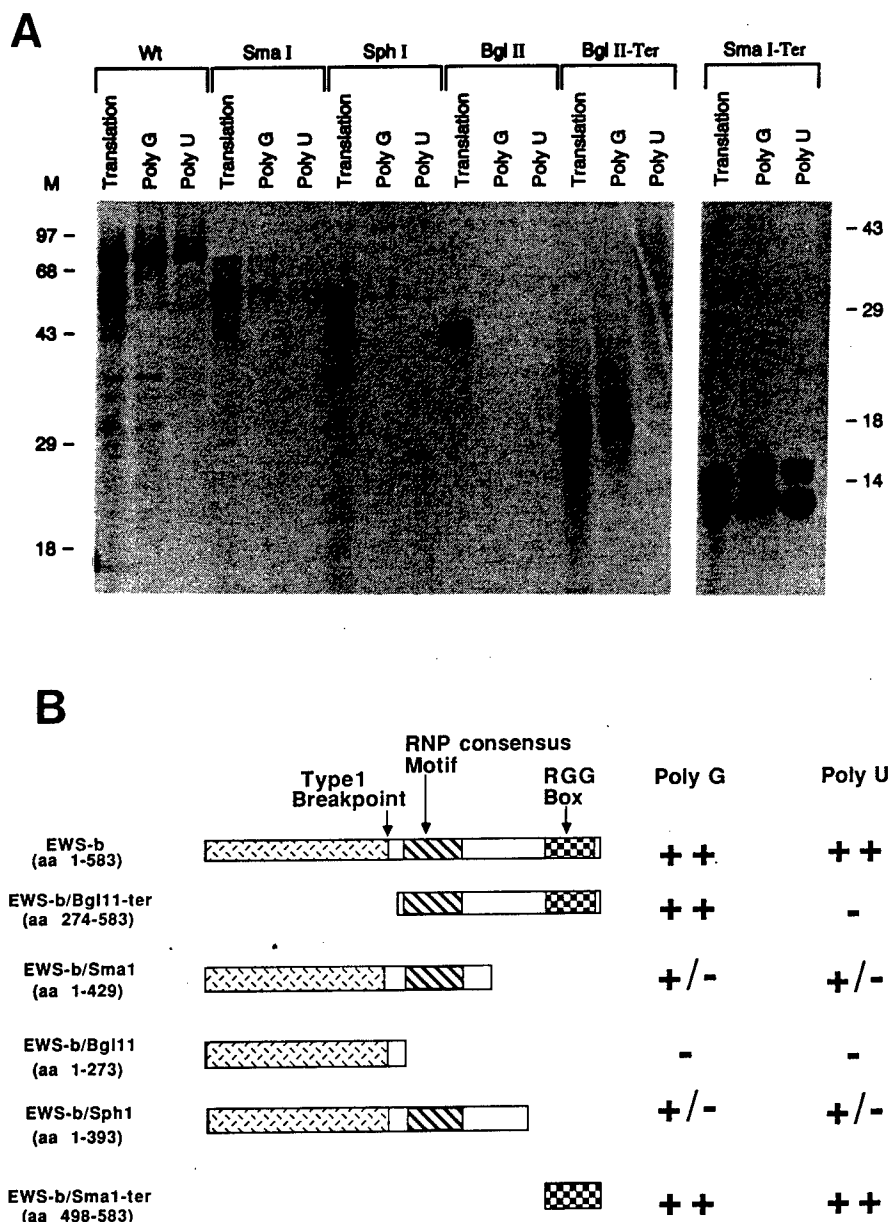


**Figure 3** (a) *In vitro* RNA binding activity of EWS-b and EWS-Fli-1 proteins. pSG5-EWS-b or pSG5-EWS-Fli-1 cDNA were linearized, *in vitro* transcribed and translated as described in Materials and Methods. These *in vitro* translated proteins were bound to the ribonucleotide homopolymers indicated, washed with 100mM NaCl, eluted with SDS sample buffer and analyzed by electrophoresis on a 12% SDS-polyacrylamide gel followed by fluorography. (b) Effect of increasing salt concentration on RNA binding activity of EWS-b protein. *In vitro* translation of EWSb protein and binding to poly (G) and poly (U) was carried out as described above. The bound EWS-b protein was washed with binding buffer containing increasing concentrations of NaCl as indicated, eluted, and characterized on SDS-PAGE as described above. (c) Single stranded DNA binding properties of EWSb and EWS-Fli-1 proteins. *In vitro* translated proteins were bound to single stranded DNA and washed with indicated salt concentrations, eluted and characterized on SDS-PAGE as described above.

estimated mass of 61 kD. The amino terminal region of EWS is rich in proline, glutamine, serine and threonine and contains multiple copies of the hexapeptide repeat (Ser/Gly-Tyr-Ser/Gly-Gln-Gln/Ser-Ser/Gln/Pro). The middle region of the EWS-b protein contains a highly conserved 85 amino-acid domain, the RMP motif or RNP-CS, found in several RNA binding proteins (Figure 1). This region is implicated in RNA binding in several RNA binding proteins (Dreyfuss *et al.*, 1988; Kenan *et al.*, 1991; Haynes,

1992), suggesting that EWS may encode an RNA binding protein. In addition, the carboxy terminal EWS domain encodes two RGG boxes, motifs that are RNA binding regions of other proteins (Kiledjian & Dreyfuss, 1992) (Figure 1). Interestingly, this middle region containing an RGG box is deleted as a result of alternative splicing.

*Expression of EWS and EWS-b transcripts in a variety of human cells*



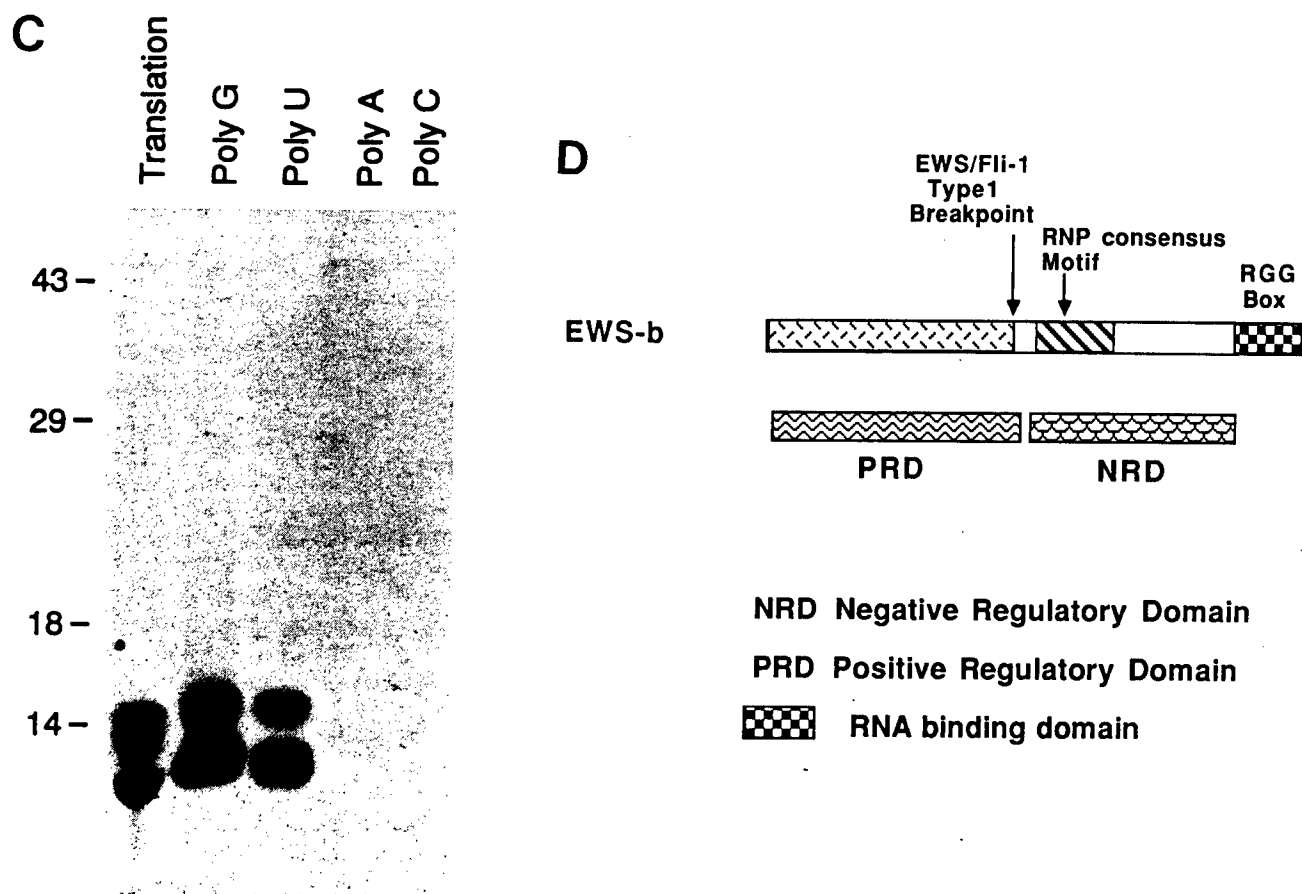
**Figure 4** (a) Localization of RNA (ribonucleotide homopolymer) -binding domain. Amino and carboxy truncated EWS-b proteins were synthesized by *in vitro* transcription and translation. These truncated proteins were tested for their ability to bind to poly (G) and poly (U) as described in figure 3. (b) Schematic representation of the structure of the above deletion mutants is shown.

Northern blot analysis of RNA from a variety of human cells reveals a heterogeneous band of EWS transcripts between 2.5-3.0 Kb (Figure 2A). This heterogeneity of the EWS transcripts may reflect differential splicing and/or differential processing of the primary EWS transcript.

We performed RNAse protection analysis to study the expression of EWS and EWS-b transcripts in different type of cells. The predicted 430 nt (corresponding to EWS-b) and 265 and 165 nt (corresponding to EWS) protected fragments were observed in Colo320 (human colon adenocarcinoma), Molt-4 (human T cell acute lymphocytic leukemia), 697 (human pre-B cell acute lymphocytic leukemia), KG-1 (human acute myelogenous leukemia) (Figure 2B). Thus both EWS and EWS-b transcripts were present in all of these cell lines.

#### *EWS gene codes for RNA binding protein*

Because both EWS encoded proteins retain the RNA binding motifs, such as RNA-CS and RGG box (Figure 1), we tested RNA binding activity of EWS-b protein. For this test, we prepared a construct with the full length EWS-b cDNA under the control of the T7 RNA polymerase promoter. This construct was linearized, *in vitro* transcribed and translated (Figure 3). The EWS-b polypeptide produced by this method migrated in SDS-polyacrylamide gels with an apparent molecular weight of approximately 80 kD, which is larger than the molecular weight (61 kD) deduced from the predicted amino acid sequence. This may have been due to the high proline content in the EWS-b protein. The *in vitro* translated EWS-b protein was assayed for



(c) RNA-binding properties of the EWS-b carboxy terminal domain. The *in vitro* translated proteins were bound to indicated ribonucleotide homopolymers, washed with 100mM NaCl, eluted with SDS sample buffer and analyzed by SDS-PAGE as described in Figure 3. (d) Schematic representation of the functional domains of EWS-b protein.

RNA binding activity using RNA homopolymers bound to agarose beads (Kiledjian & Dreyfuss, 1992). The EWS-b protein showed strong binding to both poly G and poly U, but negligible binding to poly A and poly C. Under the same conditions, *in vitro* translated EWS-Fli-1 chimeric protein failed to bind to any of the immobilized ribohomopolymers (Figure 3). We conclude from these results that EWS-b is an RNA binding protein *in vitro* and may show similar function *in vivo*.

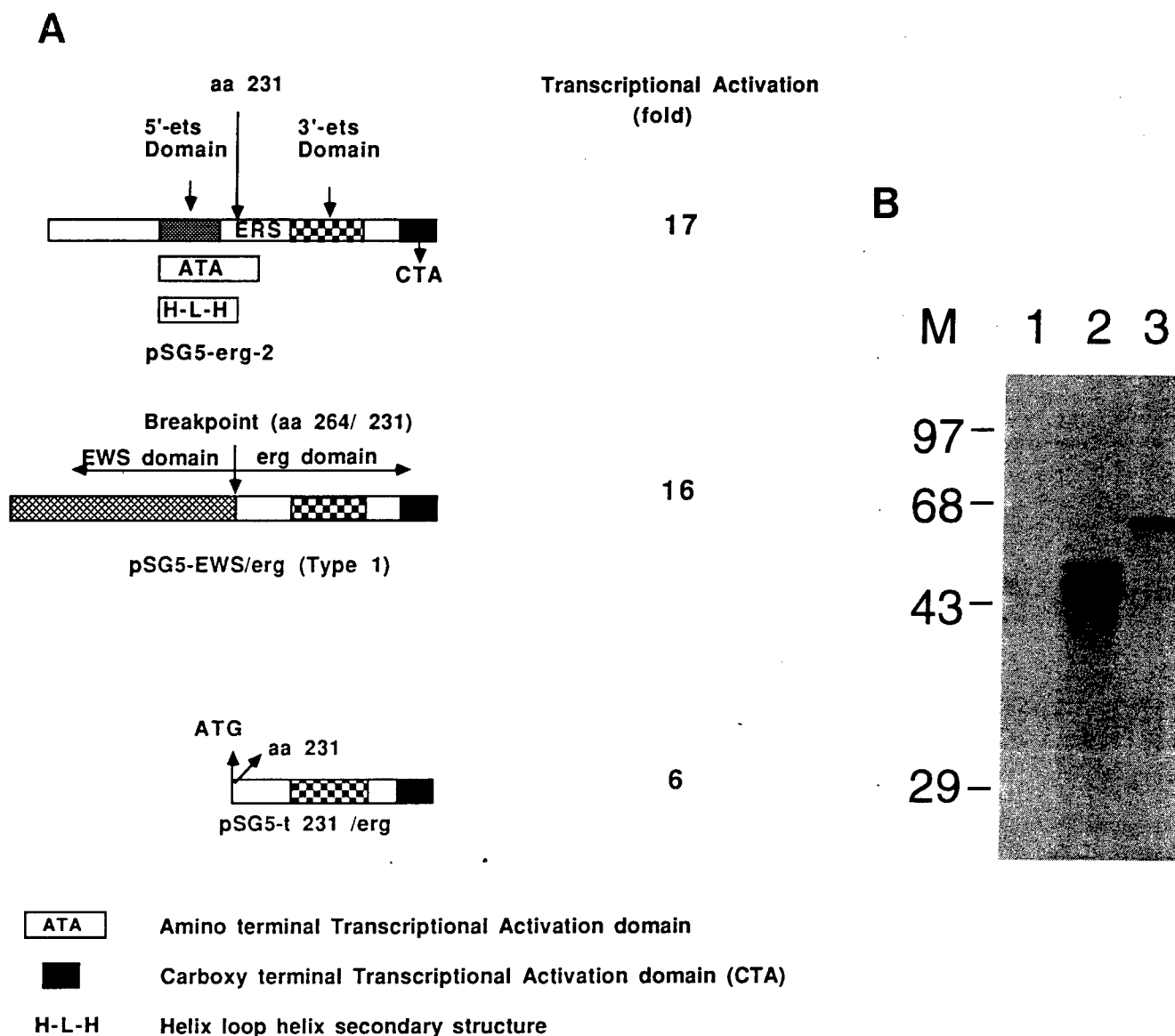
The binding of EWS-b to poly G and poly U was stable up to 0.25M NaCl concentration (Figure 3b). Stable binding of EWS-b to single stranded DNA was observed up to 0.1M NaCl concentration, suggesting that EWS-b showed stronger binding to ribopolymers (poly G and poly U) than to single stranded DNA (Figure 3c). The EWS-Fli-1 chimeric protein bound only weakly to single stranded DNA (Figure 3c).

#### Localization of the EWS domains responsible for RNA binding activity and its regulation

To determine the domain responsible for RNA binding activity and to assess the role of the conserved motifs (RNP-CS and RGG boxes) in RNA binding activity, we made a series of amino- and carboxy- truncated EWS-b by *in vitro* transcription and translation of linearized EWS-b constructs at different restriction sites. Under conditions in

which full length EWS-b protein bound well to poly G and poly U, carboxy truncated EWS-b proteins (EWS-b/Sph1 and EWS-b/Sma 1) showed weak binding, and another carboxy- truncated EWS-b polypeptide (EWS-b/Bgl 11) showed no binding to poly G and poly U (Figure 4a&b). Amino- truncated EWS-b polypeptide [EWS-b (Sma1-ter)] bound well to poly G and poly U (Figure 4a&b). This polypeptide contained the conserved RGG box suggesting that the RGG box may be required for RNA binding. Because the Sma1-ter (aa 498-583) polypeptide represents the minimal domain required for RNA binding, we tested whether this polypeptide shows binding to other ribopolymers, such as poly A and poly C (Figure 4c). The EWS-b/ (Sma1-ter) polypeptide did not bind to poly A or poly C (Figure 4c), suggesting this polypeptide also shows ribopolymer binding specificity similar to the full length EWS-b protein. The top two bands seen in the case of EWS-b/Sma1-ter may be due to usage of internal initiation codon.

Interestingly, the amino truncated EWS polypeptide EWS-b/(Bgl11-ter) showed good binding to poly G, but not to poly U (Figure 4a&b). By comparing the RNA binding activity of EWS-b/Sma1-ter and EWS-b/Bgl11-ter polypeptides (Figure 4a&b), we conclude that the domain between aa 273-498 functions as a negative regulatory domain (NRD) for the RNA binding activity (with respect to poly U binding) (Figure 4d). By comparing the RNA

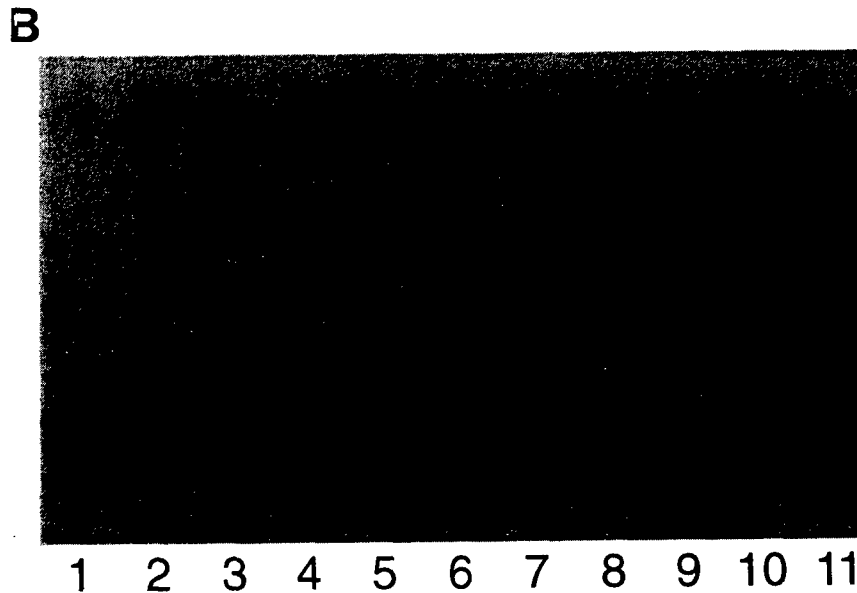
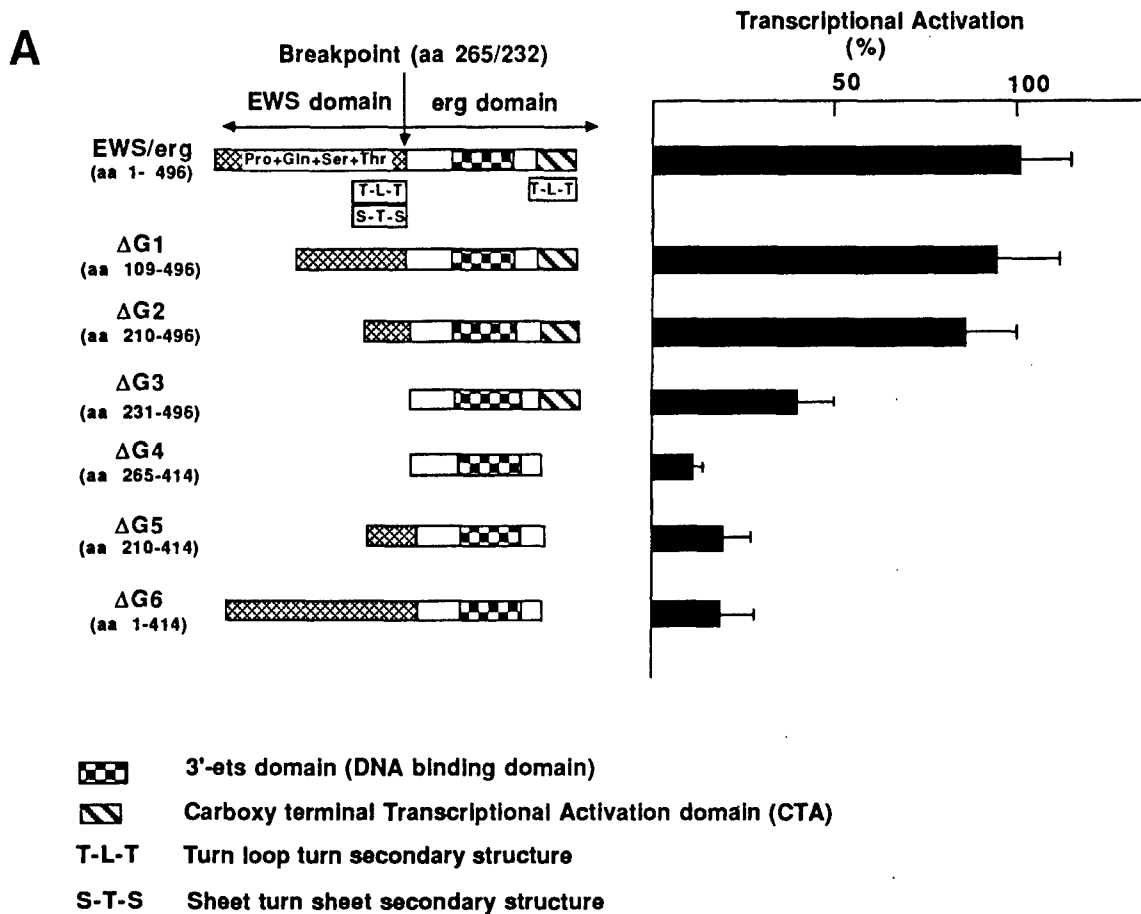


**Figure 5** (a) Schematic representation of the EWS-erg cDNA construct used for the study of transcriptional activation properties. t231/erg represents the construct, in which the amino terminal domain of EWS (NTD-EWS) is deleted from EWS-erg. Fold activation for various constructs is shown. Values represent the mean of at least five independent experiments. NIH 3T3 cells were transfected with reporter plasmid (E74-TK-CAT), reference plasmid along with each of the various expression plasmids. Cell lysates were assayed for  $\beta$ -galactosidase and CAT activities as described in materials and methods. (b) *In vitro* transcription and translation of EWS-erg and erg-2 proteins. *In vitro* transcription and translation was carried out as described in materials and methods. Lane 1, control minus RNA; lane 2, translation product of erg-2; lane 3, translation product of EWS-erg.

binding activities of EWS-b/Bgl11, EWS-b/Sph1 and EWS-b/Smal-ter, one can conclude that aa 393–498 could be responsible for negative regulation of RNA binding activity of EWS-b. Similarly, comparing the RNA binding activity of full length EWSb protein and EWSb/Bgl11-ter polypeptides (Figure 4a&b), it can be concluded that amino terminal domain of EWS-b protein (aa 1–273), may function as a positive regulatory domain (PRD) for the RNA binding activity of EWS-b protein (with respect to poly U binding) (Figure 4d). These domains are shown schematically in Figure 4d.

*EWS-erg chimeric proteins function as transcriptional activators*

The amino terminal domain of EWS (NTD-EWS) is fused to Fli-1 and erg proteins in Ewing family of tumors (Zucman *et al.*, 1993). We previously showed that this domain regulates the carboxy transcriptional activation domain of EWS-Fli-1 chimeric protein (Ohno *et al.*, 1993). To assess whether this domain also regulate EWS-erg chimeric protein function, we cloned the EWS-erg cDNA (type 1) by RT-PCR and subcloned this cDNA into SV 40 expression vector (pSG5) (Figure 5a). The EWS-erg cDNA sequence was identical (Figure 5a) to the EWS-erg cDNA sequence described earlier (Zucman *et al.*, 1993). To verify the deduced open reading frame of EWS-erg and to compare it to that of erg-2, EWS-erg and erg-2 cDNA



**Figure 6** NTD-EWS functions as a regulatory domain for the transactivation properties of EWS-erg chimeric protein. (a) Expression plasmids representing various amino- and carboxy- terminal deletions of EWS-erg were used. Each of these various expression plasmids were cotransfected with reporter and reference plasmid as described in Figure 5a. Transcriptional activation of EWS-erg protein is taken as 100%. Each bar represents the normalized values for the mean  $\pm$  SD of five independent experiments. (b) The chromatogram represents a typical transfection. lane 1, TK-CAT; lane 2, TK-CAT+ pSG-EWS-erg; lane 3, E74-TK-CAT + pSG5 vector; lane 4, E74-TK-CAT + pSG-erg-2; lane 5, E74-TK-CAT + pSG-EWS-erg; lane 6, E74-TK-CAT+pSG5-ΔG1 (aa109-496); lane 7, E74-TK-CAT+pSG5-ΔG2 (aa 210-496); lane 8, E74-TK-CAT+pSG5-ΔG3 (aa 231-496); lane 9, E74-TK-CAT+pSG5-ΔG6 (aa 1-414); lane 10, E74-TK-CAT+pSG5-ΔG5 (aa 210-414); lane 11, E74-TK-CAT+pSG5-ΔG4 (aa 265-414).



constructs were linearized, *in vitro* transcribed and translated. These *in vitro* synthesized proteins were characterized on SDS gel electrophoresis (Figure 5b). EWS-erg and erg-2 were expressed as 66kD and 52kD polypeptides, respectively, which were larger than the predicted size (Figure 5b), possibly due to high proline content of EWS-erg chimeric proteins. The top two bands that we observed with erg-2 may have resulted from usage of an internal initiation codon.

The erg proteins bind to E74 target sequences and activate the transcription of E74-TK-CAT reporter plasmid carrying three copies of E74 target sequence (Reddy & Rao, 1991, Prasad *et al.*, 1994). To test whether EWS-erg show similar transcriptional activation properties (like normal erg), we compared the transcriptional activation properties of erg-2, EWS-erg and truncated erg (devoid of EWS domain, t231/erg) (Figure 5a). Expression plasmids pSG5-erg-2, pSG5-EWS-erg and pSG5-t231/erg were cotransfected with reporter plasmid (E74-TK-CAT) and reference plasmid (pCH110). EWS-erg showed transcriptional activation with E74-TK-CAT but not with TK-CAT, demonstrating that EWS-erg codes for a sequence specific transcriptional activator (Figure 6a&b). EWS-erg and erg-2 showed similar level (16-17 fold) of transcriptional activation (Figure 5a). Deletion of NTD-EWS domain (t231/erg) from EWS-erg constructs, however, reduced transcriptional activation function (Figure 5a) suggesting that NTD-EWS is required for efficient transcriptional activation of EWS-erg chimeric protein. We previously observed two autonomous trans activation domains in the erg and Fli-1 proteins, one at the amino-terminal region (ATA domain), and the other at the carboxy-terminal region (CTA domain) (Figure 6a) (Siddique *et al.*, 1993; Rao *et al.*, 1993). In addition the H-L-H structure of the ATA domain of the erg was replaced by novel NTD-EWS (Figure 5a), resulting in novel transcriptional activation properties. Proline and glutamine amino acids were distributed throughout the NTD-EWS and such rich proline and glutamine amino acids have been shown to act as transcriptional activation domains in other transcriptional factors CTF/NF1 and Sp1 respectively suggesting that NTD-EWS may contribute trans activation domain to EWS-erg chimeric protein. Alternatively NTD-EWS may function as a regulatory/modulatory domain.

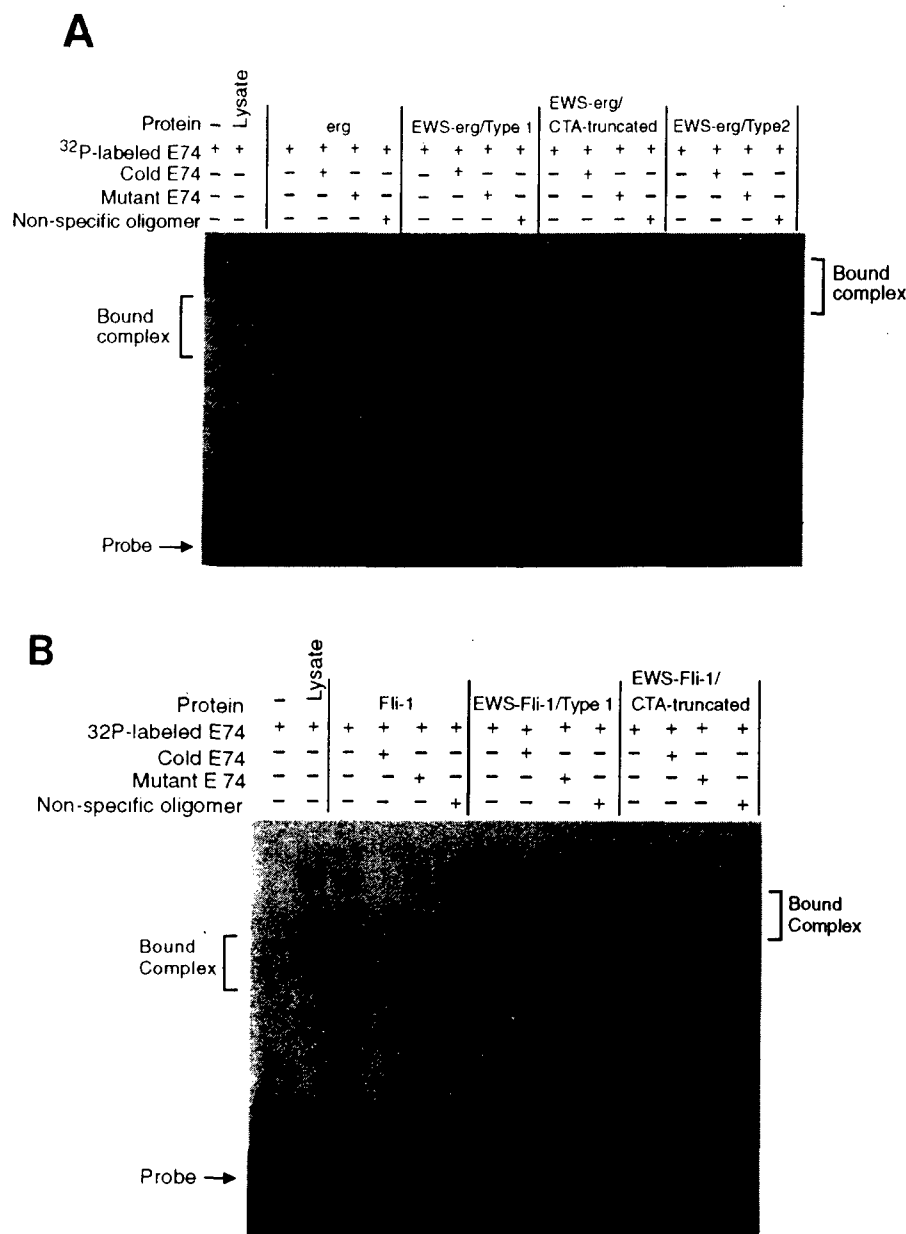
*EWS amino terminal domain (NTD-EWS) functions as a regulatory domain for the transcriptional activation function of EWS-erg chimeric protein*

To determine whether the EWS domain functions as a transcriptional activation domain or regulatory domain for the transcriptional activation properties of EWS-erg, we made a series of EWS-erg constructs that encode amino- and carboxy- deletions of EWS-erg chimeric proteins (Figure 6a), and tested them for transcriptional activation function (Figure 6a&b). EWS-erg,  $\Delta$ G1 and  $\Delta$ G2 showed similar level of transcriptional activation. Deletion of NTD-EWS in the case of  $\Delta$ G3 (aa 210 to 264), however, reduced transcriptional activation by 60%. To test the possibility that the trans activation observed in the case of EWS-erg,  $\Delta$ G1 and  $\Delta$ G2 was due to the Carboxy terminal Transcriptional Activation (CTA) domain of the erg protein (Siddique *et al.*, 1993), the CTA domain was deleted from EWS-erg constructs ( $\Delta$ G5 and  $\Delta$ G6, Figure 6a).  $\Delta$ G5 and  $\Delta$ G6 showed weak transactivation (Figure 6a&b), suggesting that the NTD-EWS domain may exert a direct

effect on transcription as well as regulation of the CTA domain of the EWS-erg chimeric protein. Previously the NTD-EWS domain was shown to function as a transcriptional activation domain (May *et al.*, 1993; Bailly *et al.*, 1994). Comparison of the trans activation function of the constructs  $\Delta$ G2,  $\Delta$ G3 and  $\Delta$ G5 suggests that aa210 to 264 of EWS domain represents the minimal EWS domain (EWS-RD) required to regulate the transcriptional activation function of EWS-erg chimeric protein. We noted a similar role for this EWS domain in the case of EWS-Fli-1 chimeric protein (Ohno *et al.*, 1993). Replacement of the Aminoterminal Trans Activation (ATA) domain of erg with regulatory domain of NTD-EWS, therefore, results in the activation of CTA domain of EWS-erg chimeric protein and may be one of the molecular mechanisms involved in these human solid tumors. The H-L-H structure of erg is replaced by EWS domain rich in glutamine, serine, tyrosine, proline and glycine. Secondary structural analysis of the EWS-RD revealed turn-loop-turn (T-L-T)/sheet-turn-sheet (S-T-S) secondary structures (Ohno *et al.*, 1993). Such T-L-T structures were observed previously in transcriptional activation domains of erg (Siddique *et al.*, 1993; Rao *et al.*, 1993), Fli-1 (Rao *et al.*, 1993) and elk-1 (Bhattacharya *et al.*, 1993).

*Effect of NTD-EWS on DNA binding properties of EWS-erg and EWS-Fli-1 chimeric proteins*

We showed that erg and Fli-1 proteins bind strongly to E74 promoter sequences in a sequence specific manner (Siddique *et al.*, 1993). To study the effect of NTD-EWS on DNA binding properties of EWS-erg chimeric protein, we compared sequence specific DNA binding properties of EWS-erg and erg proteins by Electrophoretic Mobility Shift Assay (EMSA), using *in vitro* translated EWS-erg (type 1), EWS-erg (type 2) (in this case NTD-EWS fused with DNA binding domain of erg protein) and erg-2 proteins and E74 oligonucleotide as a probe. Nucleo-protein complexes were detected for EWS-erg (type 1) (Figure 5a), EWS-erg (type 2) and erg-2 proteins with E74 sequences (Figure 7a). Two nucleo-protein complex bands seen in the case of erg protein may be due to the synthesis of two *in vitro* translated erg polypeptides as a result of usage of internal initiation codons (Figure 7a). Nucleo-protein complexes detected for EWS-erg were competed out with cold E74 oligonucleotide, but not with mutant E74 and non specific oligonucleotide (Figure 7a). These results suggest that EWS-erg (type 1 and type 2) chimeric proteins bind to DNA in a sequence specific manner (Figure 7a). Because we used equal amounts of EWS-erg and erg proteins (as measured on SDS-polyacrylamide gel and taking methionine content into consideration) and tested for DNA binding activity, our results demonstrate no dramatic differences in DNA binding activity between erg and EWS-erg proteins. We observed, however, that EWS-erg consistently showed  $\approx$  2 fold better DNA binding activity to E74 oligonucleotide compared with normal erg proteins. Competition with varying concentrations of cold E74 oligonucleotide revealed similar differences in DNA binding activities (data not shown). These results suggest that EWS-erg proteins may have modulatory DNA binding properties compared with normal erg proteins and EWS amino terminal domain may function as a regulatory/modulatory domain for the DNA binding activity of EWS-erg protein. Identification of the subtle differences between the DNA binding specificities of



**Figure 7** (a) Sequence specific DNA binding properties of EWS-erg chimeric protein. The sequence specific DNA binding activities of EWS-erg and erg proteins were carried out by Electrophoretic Mobility Shift assay (EMSA) as described in Reddy & Rao, 1991 with slight modification. *In vitro* translated EWS-erg and erg proteins were incubated with <sup>32</sup>P-labeled E74 oligomeric probe (Reddy & Rao, 1991). The bound complexes were resolved by 6% polyacrylamide gel electrophoresis and visualized by autoradiography. The proteins used were normalized based on their methionine content and intensity of the bands on SDS-PAGE. (b) Comparison of DNA binding activity of Fli-1 and EWS-Fli-1. EMSA was carried out with *in vitro* translated EWS-Fli-1 and Fli-1 proteins as described above. The nucleo-protein complexes are shown in square brackets.

EWS-erg and erg proteins may provide a clue to the activation of EWS-erg gene in the case of human solid tumors. Carboxyterminal truncated EWS-erg (aa 1-414) (EWS-erg-CTA truncated) proteins showed DNA binding activity (Figure 7a) suggesting that loss of transcriptional activation observed with EWS-erg-CTA (ΔG6 in Figure 6a) is not due to loss of DNA binding. Similar experiments with EWS-Fli-1 chimeric proteins revealed no major differences in sequence specific DNA binding properties between EWS-Fli-1 and Fli-1 proteins (Figure 7b). Carboxy-terminal truncated EWS-Fli-1 showed an approximately 2.5 fold stronger DNA binding activity as

compared with full length EWS-Fli-1 protein. Recently, an RNA binding protein gene, TLS/FUS, was found fused to the *erg* gene in human myeloid leukemia with t(16;21) chromosome translocation (Ichikawa, et al., 1994). Our unpublished results suggest that NTD-TLS/FUS (amino terminal domain of TLS/FUS) negatively regulates the DNA binding activity of TLS/FUS-erg chimeric protein and consequently transcriptional activation properties of TLS-erg chimeric protein (Ouchida *et al.*, unpublished results).

NTD-EWS may modulate the DNA binding activities and/or transcriptional activation properties of aberrant

chimeric proteins (EWS-erg or EWS-Fli-1 or EWS-ATF1 or EWS-wt1), which are found in a variety of human solid tumors. NTD-EWS may interact with tumor specific factors and modulate DNA binding activity and/or transactivation properties of chimeric proteins as occurs with elk-1 and SRF (Hipskind *et al.*, 1991; Rao & Reddy, 1992). These modulating properties affect target gene specificity and transactivation properties. S-T-S/T-L-T structures of NTD-EWS (Ohno *et al.*, 1993) may participate in these protein-protein interactions. Analysis of the target genes and the tumor specific factors that interact with EWS-erg /Fli-1/ATF1/WT1 may provide a clue to the activation of the EWS gene in these kinds of human solid tumors.

## Materials and methods

### *Molecular cloning of EWSb and EWS-erg cDNAs*

RT-PCR was performed on total RNA from Molt 4 and Ewing's sarcoma cell lines using the RT-PCR kit (Boehringer Mannheim). The cDNAs were amplified using appropriate 5' and 3' primers as described (Ohno *et al.*, 1993), subcloned, and characterized by restriction mapping and sequencing.

### *In vitro transcription and translation*

Plasmids containing appropriate cDNAs were linearized at desired restriction sites and transcribed *in vitro* with T7 RNA polymerase (Promega), according to the manufacturer's protocol. Capped RNAs were purified and translated *in vitro* using rabbit reticulocyte lysates containing [<sup>35</sup>S]methionine, according to the manufacturer's (Promega) protocol.

### *In vitro RNA and single strand DNA binding assays*

RNA binding studies, with minor modifications, were carried out as described (Siomi *et al.*, 1993). Ribonucleotide homopolymer and single strand DNA-

agarose materials were pre-equilibrated in the binding buffer [10 mM Tris-HCl pH (7.4), 2.5 mM MgCl<sub>2</sub>, 0.5% triton X-100, 100 mM NaCl]. *In vitro* translated proteins were incubated with ribonucleotide homopolymer or single strand DNA-agarose in a total volume of 0.5 ml of binding buffer (with desired NaCl concentration) for 10 min at 40°C. Agarose beads were pelleted by brief centrifugation and washed with binding buffer (with the desired salt concentration). The bound radiolabeled protein was eluted in SDS sample buffer by boiling and characterized by SDS-polyacrylamide gel electrophoresis. Protein bands were visualized by fluorography.

### *Electrophoretic mobility shift assay (EMSA)*

EMSA was carried out as described (Reddy & Rao, 1991) but with the minor modification of using *in vitro* translated proteins and E74 target sequence as a probe. The mutant and nonspecific primers used were as described (Reddy & Rao, 1991).

### *RNAase protection assay*

RNAase protection assays were performed on different RNA samples as described (Prasad *et al.*, 1994; Sambrook *et al.*, 1989). The template used for the synthesis of antisense RNA consisted of a subcloned SalI cDNA (EWS-b) fragment of 430 bp in KSM13 linearized with HindIII restriction enzyme.

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# Antisense RNA to the putative tumor suppressor gene BRCA1 transforms mouse fibroblasts

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Recently, BRCA1, a familial breast and ovarian cancer susceptible gene has been cloned and shown to be either lost or mutated in families with breast and ovarian cancers. BRCA1 has been postulated to encode a tumor suppressor, a protein that acts as a negative regulator of tumor growth. We have characterized the BRCA1 gene products by Western blot and immunoprecipitation analysis in mouse and tumor cells. Multiple BRCA1 polypeptides of approximately 225, 185, 160, 145, 100, 52 and 38 kD were identified in these cells. BRCA1 proteins were found to be localized mainly in the nucleus of normal Rat1 cells and human breast cancer cells. In order to understand the role of BRCA1 in cell transformation, we have established a stable NIH3T3 cell line expressing BRCA1 antisense RNA. The inhibition of expression of endogenous BRCA1 protein was detected in NIH3T3 transfectants by Western blot analysis. The antisense BRCA1 expressing NIH3T3 cells showed accelerated growth rate, anchorage independent growth and tumorigenicity in nude mice unlike the parental and sense transfectants. These results provide the first direct biological evidence for the possible function of BRCA1 as a tumor suppressor gene.

**Keywords:** BRCA1 proteins; antisense RNA; tumor suppressor; breast and ovarian cancers

## Introduction

Breast cancer is one of the most frequent cancers affecting women. Although majority of cases are thought to be sporadic, about 5% of cases are estimated to be familial (Claus *et al.*, 1991). The clinical progression of human breast cancer reflects accumulated molecular defects in specific genes that are important in regulating the growth of normal breast tissue. The breast cancer susceptible gene BRCA1 gene was shown to be lost or mutated in families with hereditary breast and ovarian cancers (Miki *et al.*, 1994; Takanashi *et al.*, 1995; Easton *et al.*, 1993). Some recent reports have also implicated a role for BRCA1 directly in sporadic cancers (Merajver *et al.*, 1995; Hosking *et al.*, 1995; Futreal *et al.*, 1994). Studies of allele loss in tumors from breast and ovarian cancer affected families suggesting that BRCA1 is a tumor suppressor gene (Smith *et al.*, 1992). Thus the inherited mutation results in inactivation of one copy of the gene by mutation and the loss of the second wild type allele (Smith *et al.*, 1993;

Kelsell *et al.*, 1993). These results implicate a key role for tumor suppressor genes like BRCA1 in the genesis and progression of breast cancers. The BRCA1 gene is composed of 22 coding exons stretching roughly 100 Kb of genomic DNA (Miki *et al.*, 1994). The gene codes for a 1863 amino-acid protein with an amino terminal zinc finger domain and a carboxy terminal acidic region typical of several transcriptional factors (Miki *et al.*, 1994). The first insight into the potential role of BRCA1 in breast tumor progression came from the work described by Thompson *et al.* (1995) where they show inhibition of BRCA1 expression with antisense oligonucleotides resulted in accelerated growth of normal and malignant mammary cells but not non-mammary epithelial cells. These results suggested that BRCA1 negatively regulates the proliferation of mammary epithelial cells. Taking all these results into consideration, we reasoned that if BRCA1 functions as a tumor suppressor regulating cell growth and division, allelic loss or damage by mutation of BRCA1 as seen in patients with breast cancer could result in loss of function of BRCA1 protein and uncontrolled cell growth leading to cancers. To test the hypothesis whether BRCA1 is a tumor suppressor gene, we used antisense RNA methodology (Iszat and Weintraub, 1985). We reasoned that if BRCA1 functions as a growth regulator in normal cells, inhibiting its expression should result in transformation. Our results demonstrate that antisense RNA to BRCA1 transforms mouse fibroblasts, providing the first direct biological evidence for the possible function of BRCA1, as a tumor suppressor gene.

## Results and discussion

The experimental strategy that we have used to test the hypothesis whether BRCA1 is a tumor suppressor gene is shown in Figure 1. NIH3T3 cells express significant levels of a major  $\approx 100$  kD and a minor  $\approx 145$  kD BRCA1 protein as analysed by Western blot analysis (Figure 2a, lane 1). Similar sized BRCA1 proteins were also observed in several human cell lines (Figure 2c). In addition to these bands, we have also observed both higher  $\approx 225$  kD (using nuclear extracts, data not shown), 185 kD (Figure 2c, lane 3),  $\approx 160$  kD (data not given) and lower ( $\approx 52$  and  $\approx 38$  kD) molecular weight BRCA1 polypeptides (Figure 2c). Interestingly, we have detected high level of expression of  $\approx 185$ –200 kD and  $\approx 38$  kD BRCA1 proteins in HL 60 cells by immunoprecipitation analysis (Figure 2e). These results suggest that Western blot and immunoprecipitation analysis detect different size and level of expression of BRCA1 proteins. Detection of

different size BRCA1 proteins is consistent with the presence of alternatively spliced transcripts of BRCA1 in different cells (Miki *et al.*, 1994; Rao, unpublished

results). Our results suggest that these BRCA1 proteins undergo phosphorylation (Rao, unpublished results). We have isolated and characterized a BRCA1 cDNA corresponding to  $\approx 100$  kD BRCA1 protein in human cells (Rao, unpublished results). It remains to be seen whether these multiple protein bands represent other isoforms of BRCA1 or its related proteins. We have studied the subcellular distribution of BRCA1 proteins in normal (Rat 1 cells, Figure 2d) and in transformed cells (MCF7 cells, Figure 2d) using immunohistochemical methods (Figure 2d) and indirect immunofluorescence staining (data not given). Our results suggest that BRCA1 proteins are localized mainly in the nucleus of Rat 1 and MCF7 cells. However we have also observed weak cytoplasmic staining in the case of MCF7 cells.

We have transfected NIH3T3 cells with pcDNA expression vector or pcDNA expression vectors containing antisense BRCA1 cDNA and obtained stable G418 resistant cell lines expressing antisense RNA to BRCA1. Initially we have screened several BRCA<sup>AS</sup> cell lines for BRCA1 protein expression by Western blot analysis using BRCA1 polyclonal peptide antibody. We selected two cell lines which showed a significant decrease in the expression of BRCA1 protein (Figure 2a, lanes 2 and 3). These lines showed roughly 3–5-fold decrease in the expression of BRCA1

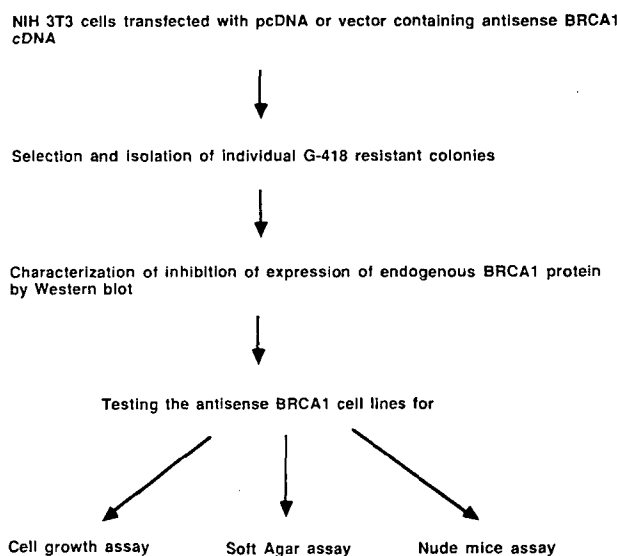
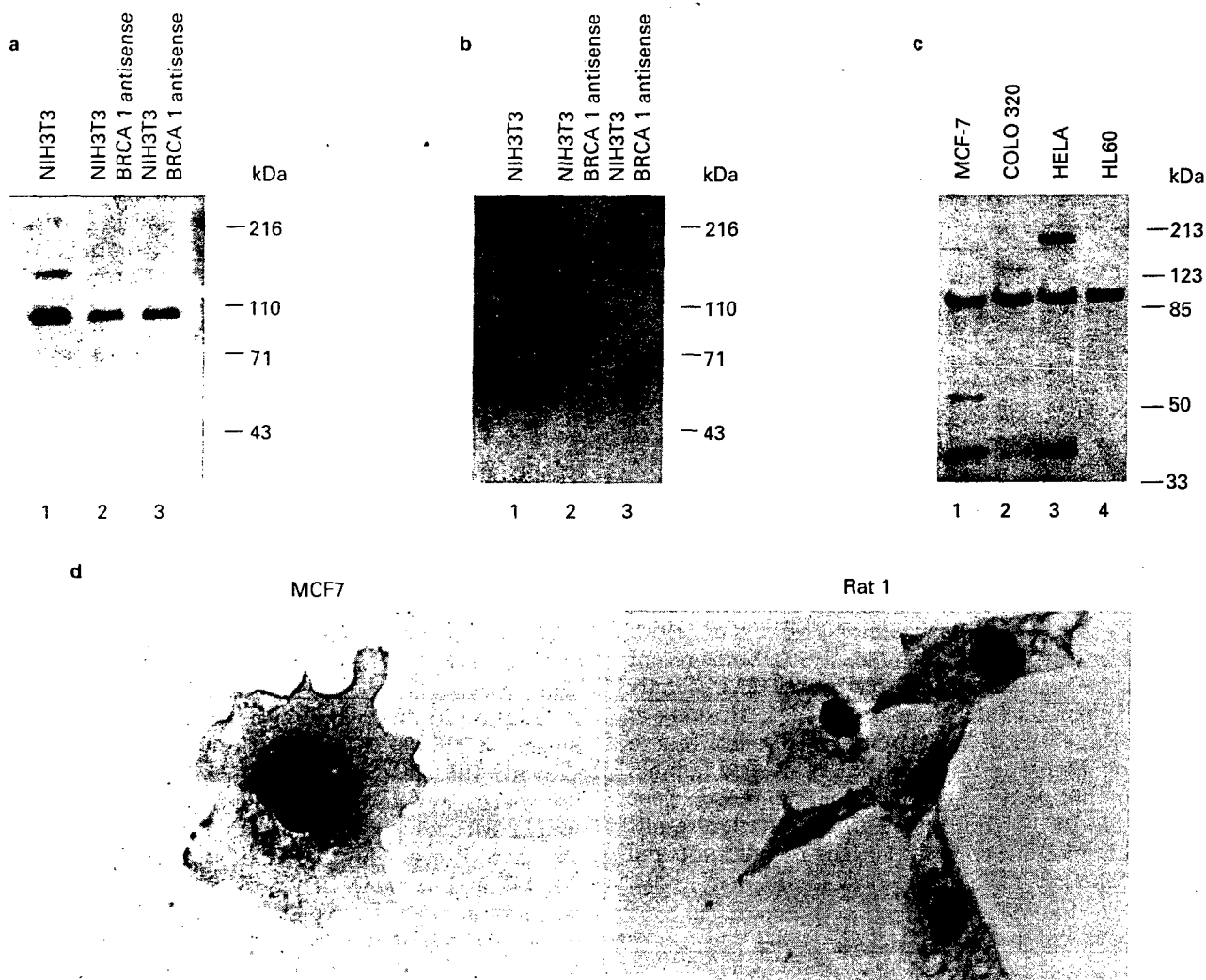


Figure 1 Experimental strategy used for obtaining the antisense BRCA1 transfectants



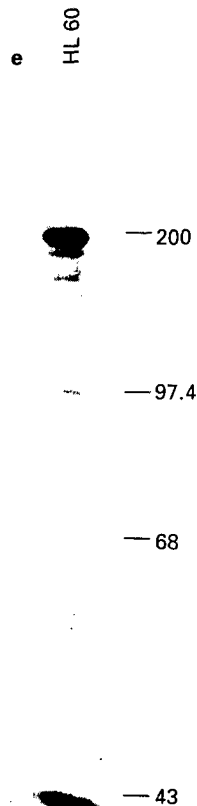
protein when compared to the parental NIH3T3 cells. We have performed BRCA1 peptide competition to show that the protein band(s) seen on the immunoblot are specific for the BRCA1 protein(s) (Figure 2b). We did not find total inhibition of expression with BRCA<sup>AS</sup> transfectants because in theory, a high concentration of antisense RNA is necessary to completely inhibit any target gene, however, our results (discussed below) suggests that total inhibition may not be necessary to observed a biological change, since mRNA molecule can synthesize several copies of protein.

The BRCA<sup>AS</sup> transfectants showed no major morphological alterations except for a slightly more flattened phenotype when compared to the parental NIH3T3 cells (compare Figure 3a and d-f). Transformed cells unlike normal cells can proliferate faster and grow in serum-free or low serum culture medium as they become independent of growth factors present in the serum. Thus we investigated the growth of BRCA<sup>AS</sup> transfectants in different serum culture conditions (10%, 0.1% and serum free). The BRCA<sup>AS</sup> transfectants proliferated at a much faster rate than NIH3T3 cells and also the BRCA<sup>AS</sup> cells proliferated in low serum media (Figure 3e and 4a and b) and serum free media (Figure 3f), whereas the parental NIH3T3 cells were unable to proliferate under these conditions (Figures 3b, c and 4b). These data indicate that BRCA<sup>AS</sup> transfectants behave like transformed cells in that they become independent of growth factors present in fetal bovine serum.

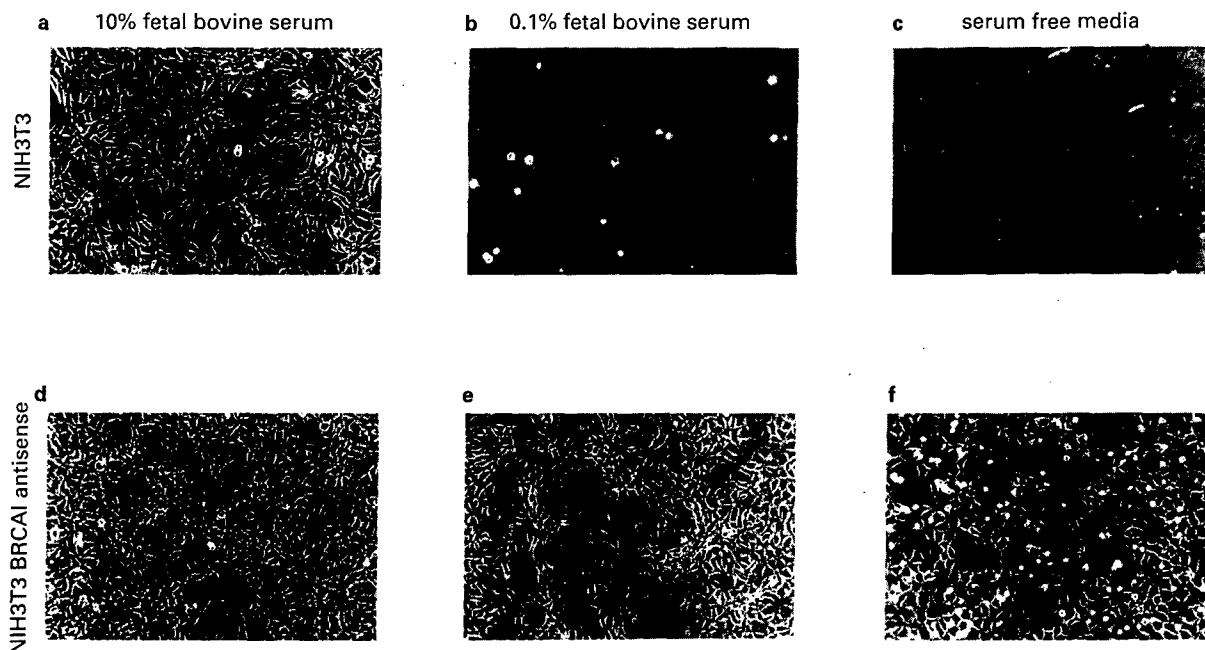
The accelerated growth rate and growth in serum free media of the BRCA<sup>AS</sup> cells raised the possibility

that they might have become transformed, hence we tested their ability to grow in soft agar. Interestingly the BRCA<sup>AS</sup> cells were anchorage independent (Figure 5a) unlike the parental NIH3T3 cells and cells transfected with the BRCA1 sense constructs (Figure 5a). BRCA<sup>AS</sup> transfectant cell line #6 showed high clonogenic affinity (average 150 colonies, Figure 5b) in soft agar assay whereas NIH3T3 cells, NIH3T3/pcDNA and NIH3T3/BRCA<sup>AS</sup> transfectants cells showed no colonies (Figure 5b). Another BRCA<sup>AS</sup> cell line number 3 also showed accelerated growth rate and growth in soft agar but was less tumorigenic than BRCA<sup>AS</sup> cell line no. 6 (data not given). Our results clearly suggests that a certain threshold level of BRCA1 protein is required for the regulation of cell growth in both mammary epithelial cells (Thompson *et al.*, 1995) and mouse fibroblasts (this study). Mere down regulation of expression of BRCA1 protein may result in deregulation of BRCA1 function leading to the progression from a normal to a transformed state. These results demonstrate that inhibition of expression of BRCA1 protein in BRCA<sup>AS</sup> cells might be sufficient to achieve transformation.

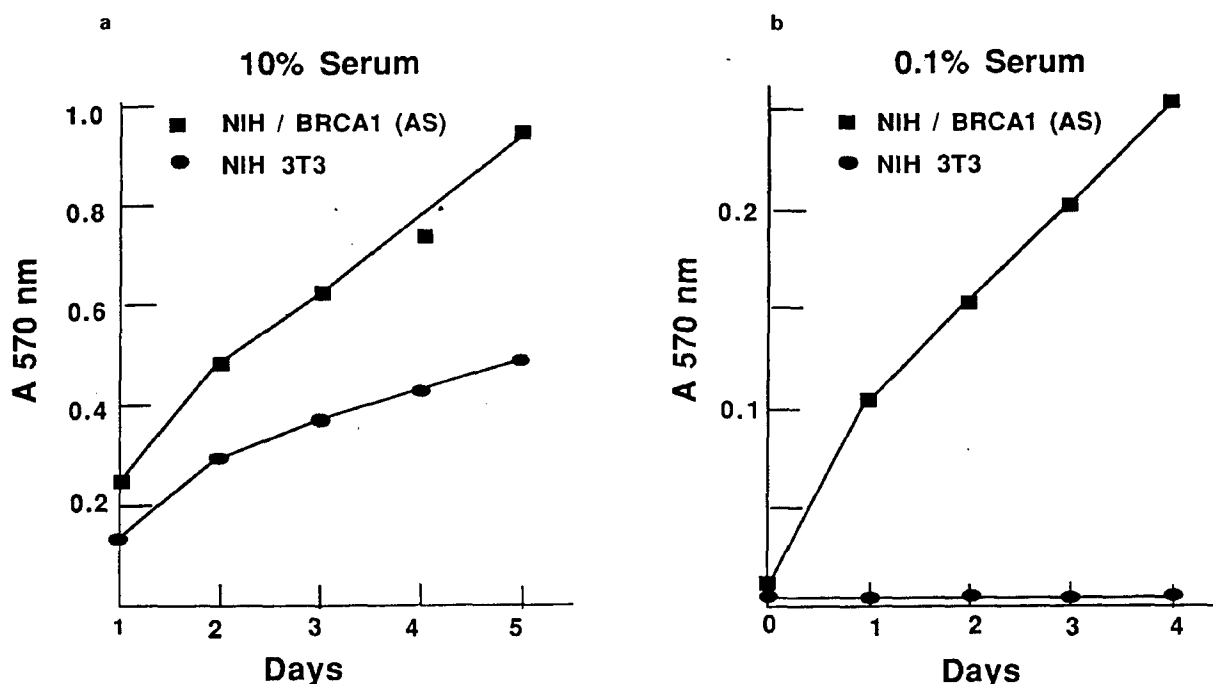
We next tested the tumorigenicity *in vivo* of NIH3T3 cells transfected with either pcDNA vector to BRCA1 antisense cDNA in nude mice. Our results show that subcutaneous injection of BRCA<sup>AS</sup> cells into nude mice consistently resulted in the development of tumors at the site of injection with a latency of 3 to 4 weeks (six out of six animals). None of the mice injected with the vector transfectants induced tumors at least up to 6 weeks.



**Figure 2** Characterization and subcellular localization of BRCA1 proteins. (a) Western blot analysis of total cellular BRCA1 protein levels in NIH3T3 and BRCA<sup>AS</sup> transfectants. Total cellular extracts from NIH3T3 cells (lane 1), BRCA1 AS cell line no 3 (lane 2) and BRCA<sup>AS</sup> cell line no 6 (lane 3) were electrophoretically separated, transferred to PVDF membrane and subjected to immunoblot analysis using a BRCA1 antipeptide antibody (Santacruz Biotechnology, Inc.). The apparent molecular weight of the prestained protein standards is shown. The molecular weight of the major BRCA1 protein band corresponds to  $\approx 100$  kD. (b) The same blot as in (a) was stripped and reprobed with the BRCA1 peptide antibody but in the presence of excess BRCA1 antigen. (c) Total cellular extract ( $\approx 70 \mu\text{g}$ ) from MCF-7 cells (human breast cancer cells) (lane 1); Colo 320 (human colon adenocarcinoma) (lane 2); HeLa cell (human epitheloid carcinoma) (lane 3); HL-60 (human promyelocytic leukemia) ( $\approx 35 \mu\text{g}$ ) (lane 4) were subjected to Western blot analysis as described in (a). (d) Immunohistochemical detection of BRCA1 proteins in normal and breast cancer cells. In Rat1 cells BRCA1 is localized only to nucleus and in MCF7 cells BRCA1 is localized mostly in nucleus. Original magnification  $\times 400$ . (e) Immunoprecipitation of BRCA1 proteins in HL60 cells. HL60 cells were labelled with [ $^{35}\text{S}$ ] trans label and subjected to immunoprecipitation using a carboxy terminal BRCA1 peptide antibody



**Figure 3** Growth properties and morphology of BRCA<sup>AS</sup> transfectants. Phase-contrast photomicrographs of NIH3T3 (a-c) and BRCA<sup>AS</sup> cell lines (d-f). Cells were cultured in normal media (10% FBS/DMEM) (a and d); low serum (0.1% FBS/DMEM) (b and e); serum free media (DMEM alone) (c and f)



**Figure 4** Inhibition of BRCA1 expression by antisense RNA accelerates NIH3T3 cell proliferation. Growth curves represent numbers of viable cells as measured by MTT dye assay (Loveland *et al.*, 1992) after different periods of cultivation in medium containing 10% FBS (a) or in 0.1% serum (b). The points represent mean of duplicates from a representative experiment

In summary, our results demonstrate that the BRCA1 gene product is a nuclear phosphoprotein which has tumor-suppressor function in mouse fibroblast cells since inhibition of endogenous BRCA1 expression by antisense RNA to BRCA1 results in neoplastic transformation. Our study provides the first direct biological evidence for the role of BRCA1 in transformation. The mechanism by which BRCA1 regulates cell proliferation is not known. The cell lines

generated in this study should be useful to study the molecular mechanism involved in the function of BRCA1 gene. Knowledge of the pathways from inhibition of BRCA1 function leading to the progression to cancer will be important for the development of diagnostic kits and for designing targeted therapeutic strategies. Future efforts will be directed towards directly testing the BRCA1 gene product for growth inhibitory function.



## Materials and methods

### Plasmid construction and transfections

cDNA of BRCA1 (V Rao and ESP Reddy, unpublished results) containing the coding exons were subcloned into pcDNA vector by PCR using the published BRCA1 primers (Miki *et al.*, 1994). Purified DNA (15 µg) of pcDNA expression vector or vector containing the sense/

antisense BRCA1 cDNAs were transfected into mouse NIH3T3 cells using the Strategene kit according to the manufacturers instructions. G418 resistant colonies were picked and propagated in the selective medium.

### Western blot analysis

Cell extracts were prepared from exponentially growing cells. The protein concentration in the extracts were determined by Bradford's method (Bio Rad) and  $\approx 18-87$  µg of protein from each of the extracts were loaded on a 10% SDS-PAGE as described previously (Rao *et al.*, 1989). After electrotransfer onto PVDF membrane, the filter was probed with a polyclonal BRCA1 peptide antibody and visualized using a chemiluminiscent assay as described by the manufacturer (Clontech kit) and exposed to Kodak X-AR film. Duplicate SDS-PAGE gels were run for each experiment and stained with coomassie blue.

### MTT dye assay

The MTT metabolic assay was performed as described (Loveland *et al.*, 1992). In brief, cells were seeded at a density of  $1 \times 10^4$  cells into microtitre plates and incubated in 10% FBS or 0.1% FBS media. At five 24 h intervals viable cells were stained for 4 h with the MTT dye and absorbance was read at 570 nm. The experiments were performed in duplicates or triplicates and reproduced at least three times.

### Anchorage independence assay

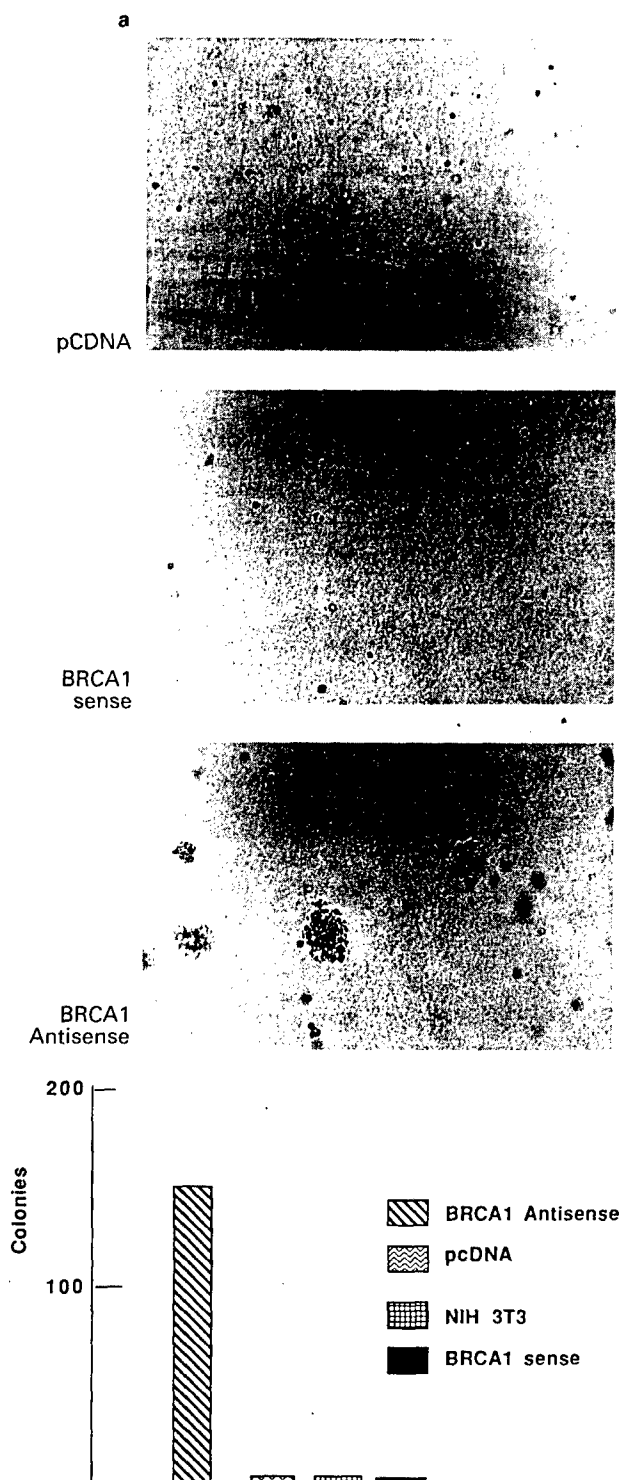
Soft agar growth assay was done in 0.3% agar/DMEM/10% FBS and plated on a base of 0.5% agar/DMEM/10% FBS. Cells were plated at a concentration of  $2 \times 10^3$  cells per 35 mm plate in soft agar containing DMEM and 10% FBS. Colonies greater than 100 µm in diameter were scored after 3 weeks. Each soft agar assay was performed in triplicates.

### Immunohistochemistry

MCF7 and Rat1 cells cultured in chamber slides were washed in PBS, fixed with 3.7% formaldehyde in PBS at room temperature for 30 min. This was followed by washes in PBS and 30 min block in blocking serum (VECTAS-TAIN, ABC system from Vector). The specimens were incubated with primary BRCA1 carboxyterminal peptide antibody diluted 1:100 at room temperature for 2 h. After washing with PBS, slides were incubated for an hour with diluted biotinylated secondary antibody solution. The slides were washed with PBS and incubated for 30 min with Vectastain ABC reagent. Slides were further washed and incubated for 5 min in 0.01%  $H_2O_2$ -0.05% DAB solution. Slides were washed for 5 min in water, mounted in cytooseal 60 (Stephens scientific) and photographed on a immunofluorescence microscope (Olympus).

### Immunoprecipitation

Briefly, confluent 100 mm plates of HL60 cells were labelled with [ $^{35}$ S] trans label for 3 h. The cells were lysed in radio immunoprecipitation assay buffer. Following sedimentation the supernatants were subjected to immunoprecipitation using rabbit anti-BRCA1 peptide antibody or preimmune serum as described previously (Rao and Reddy, 1993). The samples were subjected to 12% SDS polyacrylamide gel electrophoresis, fluorography and autoradiography.



**Figure 5** Growth of BRCA1<sup>AS</sup> cells in soft agar (a)  $2 \times 10^3$  cells per dish of pcDNA vector transfectant, BRCA1<sup>S</sup> transfectant and BRCA1<sup>AS</sup> transfectant were analysed for anchorage independent growth. (b) clonogenicity of BRCA1<sup>AS</sup> cells; pcDNA vector cells; parental NIH3T3; and BRCA1<sup>S</sup> cells

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# Induction of apoptosis by the tumor suppressor protein BRCA1

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The breast and ovarian cancer susceptibility gene BRCA1, is a nuclear phosphoprotein which functions as a tumor suppressor. To investigate the role of BRCA1 in apoptosis, we have developed mouse fibroblast cell lines and human breast cancer cell lines expressing BRCA1. The expression of BRCA1 protein in the BRCA1 transfectants were analysed by immunofluorescence and immunohistochemistry. The BRCA1 transfectants showed a flattened morphology compared to the parental cells. We show that serum deprivation or calcium ionophore treatment of BRCA1 transfectants resulted in programmed cell death. These results indicate that BRCA1 genes may play a critical role in the regulation of apoptosis. Thus, since a wide variety of human malignancies like breast and ovarian cancers have a decreased ability to undergo apoptosis, this could be due to lack/decreased levels of functional BRCA1 proteins. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 gene therapy may have the potential to prevent the progression of these malignancies.

**Keywords:** BRCA1 proteins; tumor suppressor; breast and ovarian cancers; apoptosis; therapy

## Introduction

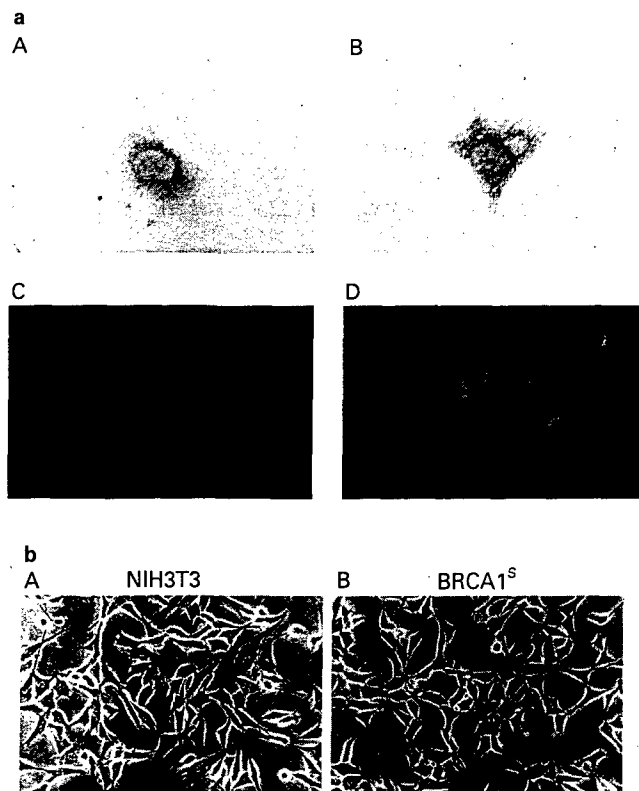
Breast cancer is one of the most common malignancy affecting women in the United States and ovarian cancer although less frequent than breast cancer is the fourth leading cause of cancer mortality among women. The breast and ovarian cancer susceptibility gene BRCA1, was recently isolated and the conceptual cDNA encodes a 1863 aminoacid protein with an amino-terminal Zinc finger domain and a carboxy terminal acidic region (Miki *et al.*, 1994). We and others have identified multiple BRCA1 proteins approximately 185-220, 160, 145, 100, 52 and 38 kD in both human and mouse cells (Rao *et al.*, 1996; Chen *et al.*, 1995). It remains to be seen whether these proteins represent isoforms of BRCA1 or its related proteins. Recently, we like others have identified alternatively spliced transcripts of the BRCA1 gene (Rao, unpublished results). A recent report indicated that the BRCA1 gene product is localized in the nucleus of several normal cell lines including breast and tumor cells other than breast and ovary (Chen *et al.*, 1995). They have detected BRCA1 mainly in the cytoplasm of almost all breast and ovarian cancer cell

lines examined. These results suggested aberrant subcellular localization of BRCA1 in breast cancer (Chen *et al.*, 1995). Interestingly, our results show BRCA1 to be localized mainly in the nucleus (or perinuclear) or cytoplasm or both of several normal or cancer cells (Rao, unpublished results) indicating variable subcellular localization of the BRCA1 proteins. Our results suggest that the subcellular localization of BRCA1 may be determined by the cell cycle status of the cells (Rao, unpublished results). Our results show that BRCA1 interacts with cyclin dependent kinases suggesting a role for BRCA1 in cell cycle regulation (Rao, unpublished results). We have previously reported that the BRCA1 gene product to be a nuclear protein with tumor suppressor function in mouse fibroblast cells since inhibition of endogenous BRCA1 expression by antisense RNA to BRCA1 resulted in neoplastic transformation (Rao *et al.*, 1996). We have recently observed that introduction of variant BRCA1 gene into human cancer cells results in suppression of growth and neoplastic phenotype (Rao *et al.*, unpublished results) implicating a direct role for BRCA1 in growth and tumor suppression.

## Results and discussion

To study the function of BRCA1 genes in the regulation of apoptosis, we have transfected NIH3T3 cells with pcDNA expression vector or pcDNA expression vector containing human BRCA1 cDNA and obtained stable G418 resistant cell lines expressing BRCA1. These BRCA1<sup>s</sup> cell lines were analysed for BRCA1 protein expression by indirect immunofluorescence analysis (Figure 1a) and immunoperoxidase staining (Figure 1a) using BRCA1 polyclonal antibody as described previously (Rao *et al.*, 1996). The nuclear and cytoplasmic staining was brighter and stronger in BRCA1<sup>s</sup> transfectants compared to parental NIH3T3 cells (Figure 1a). The morphology of the BRCA1<sup>s</sup> transfectants were different from that of the parental NIH3T3 cells. The BRCA1<sup>s</sup> cells are shorter and flatter when compared to the parental NIH3T3 cells (Figure 1b) and with several weeks in continuous culture the BRCA1<sup>s</sup> cells become spindle shaped with elongated processes leading to their detachment. These results suggest that constitutive high level expression of the BRCA1 gene product for a prolonged period of time may result in apoptosis.

One of the most efficient ways of triggering the apoptotic response in fibroblasts is the removal of serum (Jimenez *et al.*, 1995). Thus we next investigated the effect of serum withdrawal on the induction of apoptosis in the BRCA1<sup>s</sup> transfectants and compared it with that of the NIH3T3 cells. Subconfluent NIH3T3 and BRCA1<sup>s</sup> cells were grown in medium containing



**Figure 1** Detection of BRCA1 protein(s) in NIH3T3 and BRCA1<sup>s</sup> cells by immunohistochemically and immunofluorescence analysis. (a) Immunoperoxidase (A, B) and immunofluorescence (C, D) analysis. A, NIH3T3; B, BRCA1<sup>s</sup>; C, NIH3T3; D, BRCA1<sup>s</sup>. (b), Morphology of the BRCA1<sup>s</sup> transfectants. Phase-contrast photomicrographs of NIH3T3 (A) and BRCA1<sup>s</sup> cell lines cultured in normal media (10% FBS/DMEM)

either 10% or 0% fetal bovine serum (FBS). After 24 h the cells were collected and subjected to flow cytometric analysis. The BRCA1<sup>s</sup> transfectant cells showed enhanced rates of apoptosis under serum depleted conditions, as measured by the appearance of an additional 'sub G1' peak on flow cytometry (Figure 2a). This peak is associated with high levels of DNA degradation. High levels of apoptotic cells in the sub G<sub>0</sub>/G1 peak (A<sub>p</sub>) population were measured in BRCA1<sup>s</sup> cells (A<sub>p</sub> value 75%) whereas the control cell line NIH3T3 showed lower levels of apoptosis under identical conditions (Figure 2a). BRCA1<sup>s</sup> cells did not show any significant apoptosis levels when grown under normal conditions of DMEM supplemented with 10% FBS (Figure 2a).

The above results of apoptosis in BRCA1<sup>s</sup> transfectants was further confirmed by DNA fragmentation assay. Here NIH3T3 and BRCA1<sup>s</sup> transfectant cells were cultured in serum free media for 24, 48 and 72 h and then analysed for DNA fragmentation in agarose gels. The BRCA1<sup>s</sup> transfectant cell line showed the production of a typical oligonucleosomal DNA ladder (Figure 2b; B, lanes 1–3) indicating activation of apoptosis. This effect was readily seen for BRCA1<sup>s</sup> cell lines cultured in serum free media but not for the parental NIH3T3 cells grown under identical conditions (Figure 2b; A, lanes 1 to 3). These results indicate that over expression of BRCA1 accelerates apoptosis in serum depleted NIH3T3 cells.

Apoptosis can be induced by calcium ionophore, A23187, in thymocytes (McConkey *et al.*, 1989). This

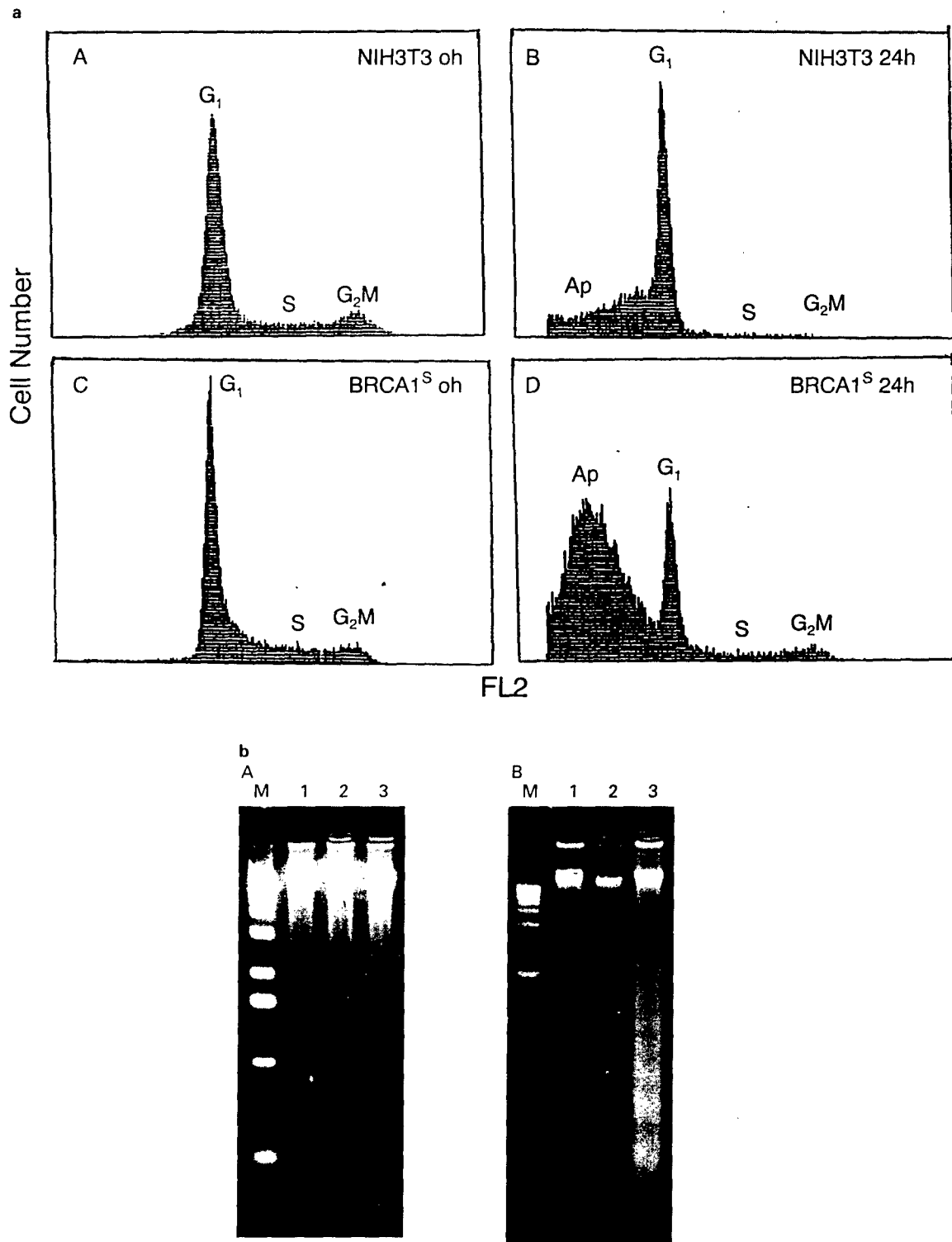
led us to examine apoptosis in BRCA1<sup>s</sup> cells during A23187 treatment. NIH3T3 and BRCA1<sup>s</sup> cells were treated with calcium ionophore A23187 for 24 h and the cell cycle distribution was determined by flow cytometry with propidium iodide staining method (Bendall *et al.*, 1994). Histogram of the DNA content and the percentage of cells in G1, S and G2 plus M of the cell cycle were evaluated by computer analysis using EPICS profile analyzer. The BRCA1<sup>s</sup> transfectant cells showed accelerated rates of apoptosis (A<sub>p</sub> value 82%) in presence of calcium ionophore (Figure 3a). Measurement of apoptosis through the sub G1 peak in the DNA histogram has the advantage of simplicity. But it has the disadvantage that since all the cells are fixed there is no distribution between viable and dead cells. Therefore the viability of both NIH3T3 cells and BRCA1<sup>s</sup> cells cultured in the presence of calcium ionophore was tested by crystal violet staining. Cell viability staining showed that majority of the BRCA1<sup>s</sup> cells treated with calcium ionophore A23187 were dead whereas most of the control NIH3T3 cells survived (Figure 3). These results suggest that BRCA1 induces death in NIH3T3 cells.

Apoptosis in the BRCA1<sup>s</sup> transfectants was further confirmed in two ways. First, NIH3T3 and BRCA1<sup>s</sup> cells were cultured in the presence of calcium ionophore to induce apoptosis and the incidence of cell death was determined by phase contrast microscopy after staining the cultures with Hoechst 33258 (Oberhammer *et al.*, 1994). Majority of the nuclei of BRCA1<sup>s</sup> showed strong chromatin condensation and nuclear degradation into small, spherical nuclear particles of condensed chromatin characteristic of apoptosis (Figure 3c), whereas the parental NIH3T3 cells did not show any significant change in the staining pattern (Figure 3). Second, the analysis of DNA degradation upon treatment of BRCA1<sup>s</sup> transfectants with calcium ionophore confirmed induction of apoptosis. Figure 3d shows that the DNA of BRCA1<sup>s</sup> cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder whereas the parental NIH3T3 cells had no significant DNA degradation (Figure 3d). These results suggested that calcium ionophore induces apoptosis more readily in BRCA1<sup>s</sup> cells than NIH3T3 cells. All the above results shown for one clone of NIH3T3–BRCA1<sup>s</sup> cells, have been reproducibly obtained with several other independent clonal isolates of NIH3T3–BRCA1<sup>s</sup> cell lines (data not shown).

In an attempt to understand the role of BRCA1 genes in the regulation of apoptosis of human breast cancer cells, we have transfected MCF7 cells with pcDNA expression vector or pcDNA expression vector containing human BRCA1 cDNA and obtained stable G418 resistant cell lines expressing BRCA1 (Rao *et al.*, unpublished results). These BRCA1<sup>s</sup> cell lines were analysed for BRCA1 protein expression by indirect immunofluorescence analysis, immunoperoxidase staining and Western blot analysis (Rao *et al.*, unpublished results). Apoptosis in the MCF-7 BRCA1<sup>s</sup> transfectants were analysed after treatment with calcium ionophore A23187. MCF-7 and BRCA1<sup>s</sup> cells were treated with calcium ionophore A23187 for 24 h and the cell cycle distribution was determined by Flow cytometry with propidium iodide staining. The BRCA1<sup>s</sup> transfectant MCF-7 cells showed accelerated

rates of apoptosis ( $A_p$  value 75%) in the presence of calcium ionophore (Figure 4a). The viability of both MCF-7 cells and BRCA1<sup>s</sup> cells cultured in the presence of calcium ionophore was tested by crystal violet staining. Cell viability staining showed that a vast majority of the MCF-7 BRCA1<sup>s</sup> cells treated with calcium ionophore A23187 were dead whereas most of the control MCF-7 cells survived (Figure 4b). These results suggest that BRCA1 induces death in MCF-7

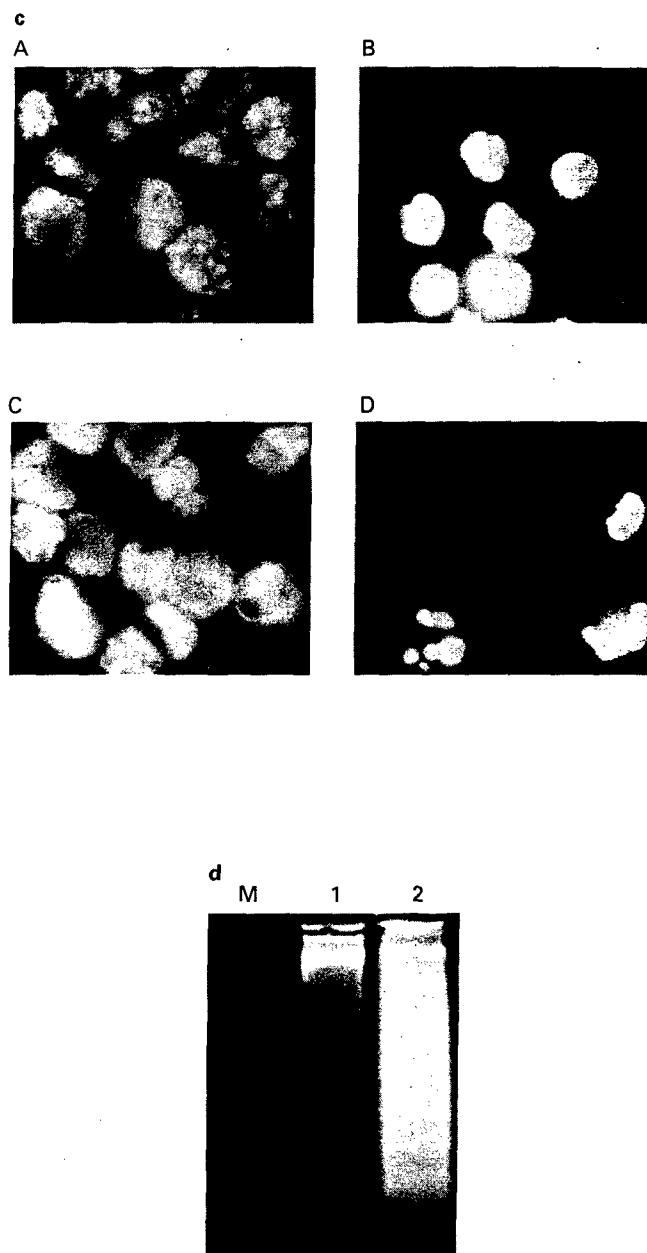
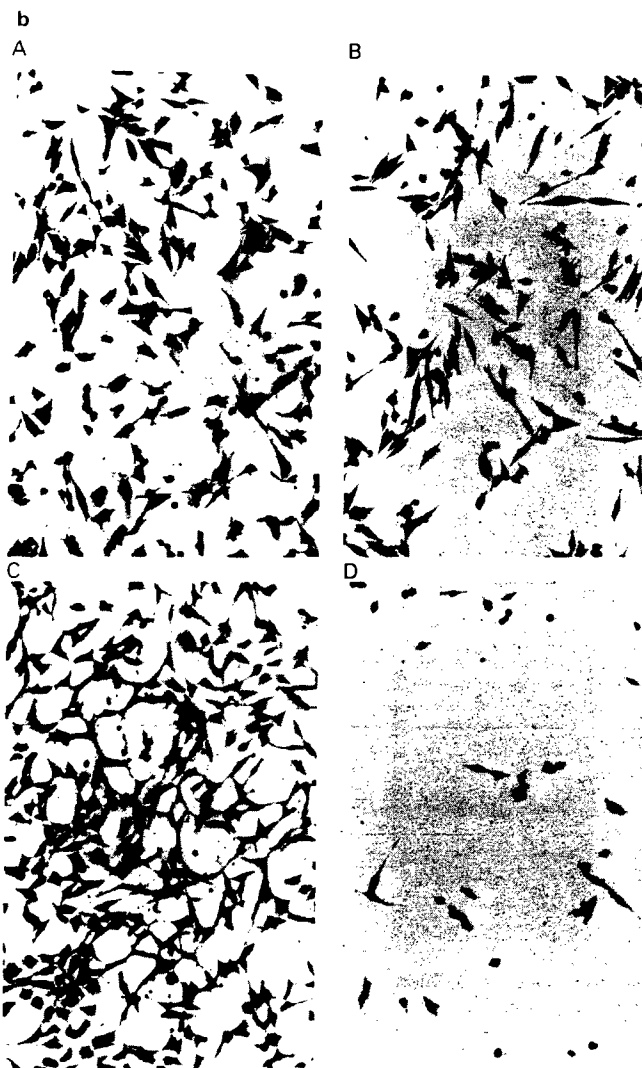
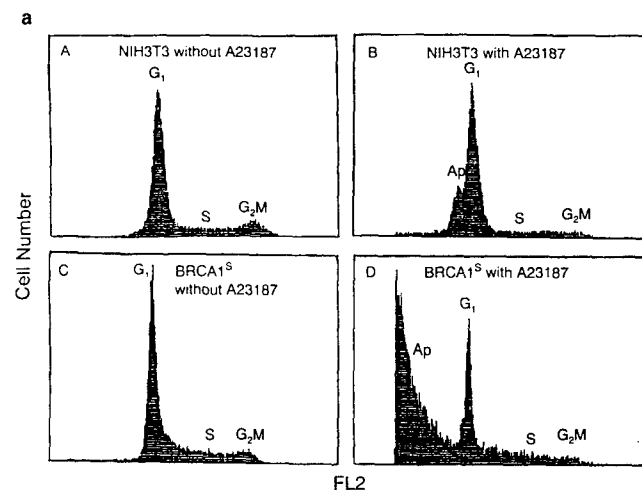
cells. The induction of apoptosis in the BRCA1<sup>s</sup> transfectants was further confirmed by analysis of DNA fragmentation upon treatment of BRCA1<sup>s</sup> transfectants with calcium ionophore. The DNA of BRCA1<sup>s</sup> cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder unlike the parental MCF-7 cells (Figure 4c). These results suggest that over expression of BRCA1 in breast cancer cells results in apoptosis.



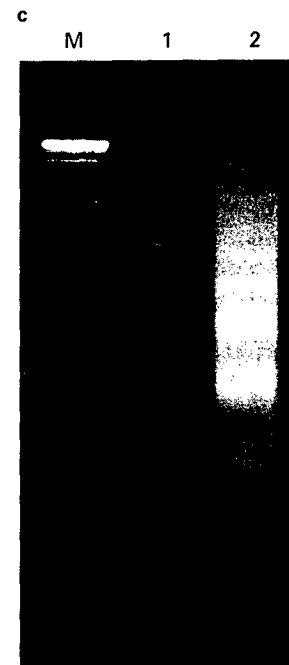
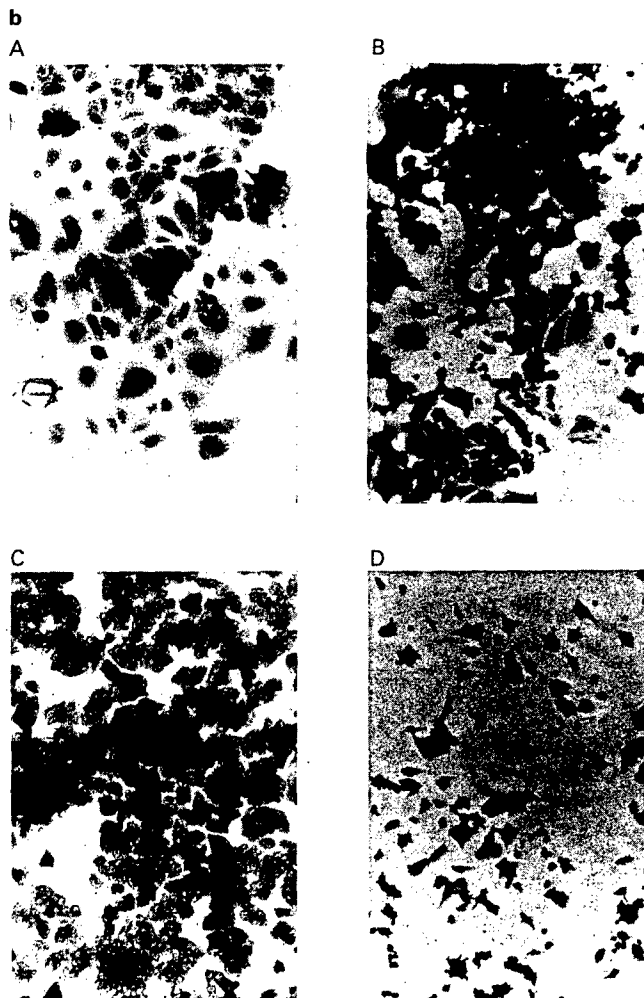
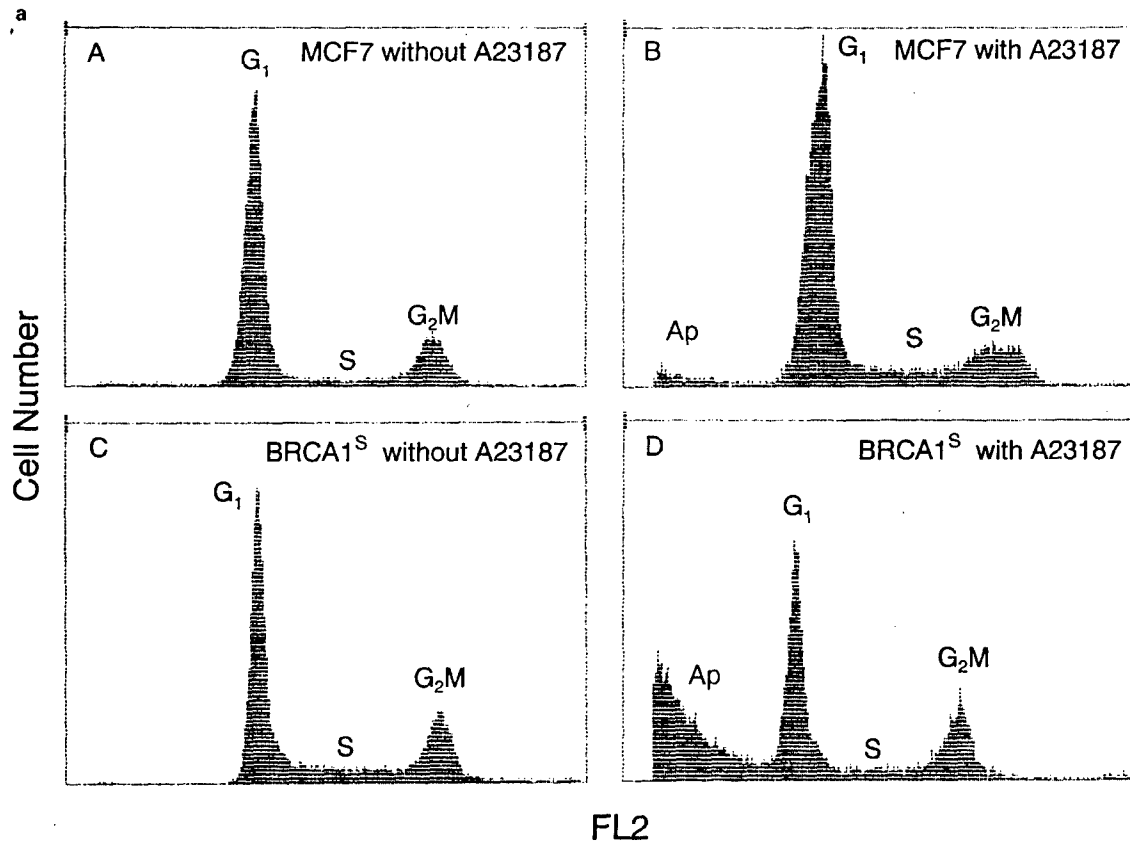
**Figure 2** BRCA1 over expression induces apoptosis in serum depleted NIH3T3 cells. (a) Flow cytometric analysis of cells induced to undergo apoptosis by serum deprivation. A, NIH3T3, 0 h; B, NIH3T3, 24 h; C, BRCA1<sup>s</sup> 0 h; D, BRCA1<sup>s</sup> 24 h. (b) Over expression of BRCA1 gene induces DNA fragmentation typical of apoptosis in serum deprived cells. A, control NIH3T3 cells serum starved for 24 h (lane 1); 48 h (lane 2); 72 h (lane 3) and B, BRCA1<sup>s</sup> cell serum starved for 24 h (lane 1); 48 h (lane 2); 72 h (lane 3)

In summary, our results demonstrate that the BRCA1 gene product which is a nuclear phosphoprotein (Rao *et al.*, 1996; Chen *et al.*, 1995) with tumor suppressor properties (Rao *et al.*, 1996; Rao *et al.*, unpublished results) functions as an inducer of apoptosis similar to rho, c-myc, p53, E1A and rel (Jimenez *et al.*, 1995; Fisher, 1994; Hoffman and Libermann, 1994; Yonish-Rouach *et al.*, 1991). The BRCA1 cDNA used in this study lacks

majority of exon 11, suggesting that this region of exon 11 is dispensable for the apoptotic function of BRCA1. The precise mechanism by which BRCA1 triggers cell death remains to be investigated. It may be possible that BRCA1 gene products function as transcriptional regulators that may either activate death inducing genes or repress death inhibiting genes leading to apoptosis. Alternatively, BRCA1 may activate apoptosis inducing



**Figure 3** BRCA1 induces apoptosis in NIH3T3 cells after calcium ionophore treatment. (a) Flow cytometric analysis of cells induced to undergo apoptosis by calcium ionophore A23187 treatment for 24 h. (b) Viability of NIH3T3 and BRCA1<sup>S</sup> cells treated with and without calcium ionophore for 24 h. A, NIH3T3; B, NIH3T3 treated with calcium ionophore; C, BRCA1<sup>S</sup>; D, BRCA1<sup>S</sup> treated with calcium ionophore. (c) Chromatin condensation shown morphologically by Hoeschst staining of the BRCA1<sup>S</sup> cell line induced to undergo apoptosis by calcium ionophore treatment for 24 h. A, NIH3T3 in the absence of calcium ionophore; B, NIH3T3 in the presence of calcium ionophore; C, BRCA1<sup>S</sup> in the absence of calcium ionophore; D, BRCA1<sup>S</sup> in the presence of calcium ionophore. (d) DNA fragmentation induced by BRCA1 overexpression. 1, NIH3T3 treated with calcium ionophore; 2, BRCA1<sup>S</sup> treated with calcium ionophore



**Figure 4** BRCA1 over expression induces apoptosis in MCF-7 cells after calcium ionophore treatment. (a) Flow cytometry analysis of cells induced to undergo apoptosis by calcium ionophore A23187 treatment for 24 h. (b) Cell viability of MCF-7 and BRCA1<sup>S</sup> cells treated with and without calcium ionophore for 24 h. A, MCF-7; B, MCF-7 cells treated with calcium ionophore; C, BRCA1<sup>S</sup>; D, BRCA1<sup>S</sup> treated with calcium ionophore. (c) DNA fragmentation induced by BRCA1 over-expression. Lane 1, MCF-7 cells treated with calcium ionophore; 2, BRCA1<sup>S</sup> cells treated with calcium ionophore

proteins or target apoptosis inhibiting proteins through direct protein-protein interactions. In the mouse mammary gland BRCA1 expression was found to be elevated during pregnancy following treatment with ovarian hormones (Lane *et al.*, 1995; Marquis *et al.*, 1995) and in human breast cancer cells BRCA1 mRNA levels were found to be regulated by steroid hormone estrogen and progesterone (Gudas *et al.*, 1995). Recent evidence suggests that hormone-dependent tumors like breast and ovarian cancers have a decreased ability to undergo apoptosis (Thompson, 1995). Our results suggest that lack or decreased levels of expression of functional BRCA1 gene product in breast and ovarian cancers may be responsible for the increased resistance of these cells to undergo apoptosis. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 gene therapy may have the potential to prevent the progression of these malignancies. Alternatively, one can use therapeutic agents that can activate BRCA1 downstream signals involved in apoptosis for the treatment of breast and ovarian cancers.

## Materials and methods

### Cell lines

NIH3T3 cells stably overexpressing the BRCA1 gene product has been previously described (Rao *et al.*, 1996). Cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-streptomycin and 200 µg/ml of G418 under 5% CO<sub>2</sub> atmosphere. MCF-7 cells were transfected with BRCA1 cDNA as described previously (Rao *et al.*, 1996; Rao *et al.*, unpublished results).

### Immunohistochemistry

NIH3T3 cells stably overexpressing the BRCA1 gene product has been previously described (Rao *et al.*, 1996). Cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-streptomycin and 200 µg/ml of G418 under 5% CO<sub>2</sub> atmosphere. NIH3T3 and BRCA1<sup>s</sup> transfectant cells were cultured in chamber slides and processed for immunohistochemistry using the BRCA1 peptide antibody as described previously (Rao *et al.*, 1996).

### Immunofluorescence

Immunofluorescence analysis was done as described previously (Dyck *et al.*, 1994). In brief, NIH3T3 and BRCA1<sup>s</sup> cells cultured in chamber slides were fixed in 3.7% formaldehyde at room temperature, following by washing with PBS and blocking in blocking solution (3% bovine serum albumin-0.5% Triton X-100 in PBS) for 30 min at room temperature. The cells were incubated with primary BRCA1 peptide antibody diluted 1:100 for 2 h at room temperature, then washed three times with PBS and were incubated with the secondary antibody (FITC conjugated goat anti rabbit IgG (Cappel). After rinsing in PBS, the slides were mounted with fluorescence mounting media (Vector) and photographed on an immunofluorescence microscope.

### Flow cytometry analysis

Subconfluent to confluent NIH3T3, BRCA1<sup>s</sup> cells were incubated in 10% FBS or 0% FBS media. After 24 h both adherent and nonadherent cells were pooled, washed in PBS and fixed in 80% cold ethanol at -18°C overnight. Cells were pooled, washed in PBS and stained with

propidium iodide (20 µg/ml) and incubate with 20 µg/ml of RNase A at 4°C in the dark overnight. Samples were analysed using a EPICs profile analyzer. Histograms showing the total DNA content at FL2 vs cell number are shown.

Flow cytometry analysis of NIH3T3, MCF7 and BRCA1<sup>s</sup> cells treated with calcium ionophore A23187 was done similar to that described in Figure 2 legend except for the treatment with 20 µM calcium ionophore A 23187 (Sigma) for 24 h.

### DNA fragmentation

DNA fragmentation assay was done as described (Kondo *et al.*, 1995). In brief, confluent NIH3T3 and BRCA1<sup>s</sup> cells grown in DMEM supplemented with 10% FBS and penicillin streptomycin were changed into media containing 0% FBS for 24, 48 and 72 h. After the indicated incubation periods, both adherent and detached cells were collected (2–5 × 10<sup>6</sup> cells), washed once in TBS buffer and lysed in 1 ml of 100 mM Tris-HCl, 0.1 M EDTA, 0.5% SDS and 20 µg/ml RNase A (pH 8.0) and incubated at 37°C for 30 min. Proteinase K at a final concentration of 100 µg/ml was added and further incubated for 3 h at 55°C. After extraction with an equal volume of phenol:chloroform: isoamyl alcohol, followed by re-extraction with phenol:chloroform: isoamyl alcohol. The DNA was precipitated from the aqueous phase with sodium acetate and two volumes of ethanol, the DNA pellet was dissolved in TE buffer and analysed by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining.

DNA fragmentation analysis of NIH3T3, MCF7 and BRCA1<sup>s</sup> cells treated with calcium ionophore A23187 was done as described in Figure 2 legend except for the treatment of A23187 (20 µM) for 24 h before analysis on a 2% agarose gel.

### Cell viability

Subconfluent NIH3T3, MCF-7-BRCA1<sup>s</sup> cells seeded into six well plates and grown in DMEM 10% FBS were treated with 20 µM calcium ionophore A23187 for 24 h. After which cells were washed with PBS and fixed in methanol:glacial acetic (3:1). Cells were stained with crystal violet. After washing in water, the cells were visualized and photographed under an Olympus microscope at 200 × magnification.

### Chromatin condensation

NIH3T3 and BRCA1 cells treated with or without 20 µM calcium ionophore for 24 h were cultured on glass cover slips and fixed in methanol:glacial acetic acid (3:1) at -18°C for 30 min. The cells were washed in PBS and stained with 8 µg/ml Hoechst 33258 for 5 min in dark. The coverslips were rinsed in water and mounted with fluorescence mounting media. The cells were visualized and photographed under the fluorescence microscope.

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# BRCA1 proteins are transported to the nucleus in the absence of serum and splice variants BRCA1a, BRCA1b are tyrosine phosphoproteins that associate with E2F, cyclins and cyclin dependent kinases

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BRCA1, a familial breast and ovarian cancer susceptibility gene encodes nuclear phosphoproteins that function as tumor suppressors in human breast cancer cells. Previously, we have shown that overexpression of a BRCA1 splice variant BRCA1a accelerates apoptosis in human breast cancer cells. In an attempt to determine whether the subcellular localization of BRCA1 is cell cycle regulated, we have studied the subcellular distribution of BRCA1 in asynchronous and growth arrested normal, breast and ovarian cancer cells using different BRCA1 antibodies by immunofluorescence and immunohistochemical staining. Upon serum starvation of NIH3T3, some breast and ovarian cancer cells, most of the BRCA1 protein redistributed to the nucleus revealing a new type of regulation that may modulate the activity of BRCA1 gene. We have also characterized two new variant BRCA1 proteins (BRCA1a/p110 and BRCA1b/p100) which are phosphoproteins containing phosphotyrosine. Immunofluorescence and Western blotting analysis indicate cytoplasmic and nuclear localization of BRCA1a and BRCA1b proteins. To elucidate the biological function of BRCA1, we created a bacterial fusion protein of glutathione-transferase (GST) and BRCA1 zinc finger domain and detected two cellular proteins with molecular weights of approximately 32 and 65 kD, one of which contains phosphotyrosine designated p32 and p65 BRCA1 interacting proteins (BIP) that specifically interact with BRCA1. Western blot analysis of BIP with cyclins/CDKs and E2F antisera indicated association with cdc2, cdk2, cdk4, cyclin B, cyclin D, cyclin A and E2F-4 but not with cdk3, cdk5, cdk6, E2F-1, E2F-2, E2F-3, E2F-5 and cyclin E. Furthermore, we have also demonstrated a direct interaction of *in vitro* translated BRCA1a and BRCA1b proteins with recombinant cyclin A, cyclin B1, cyclin D1, cdc2, cdk2 and E2F fusion proteins *in vitro*. Taken together these results seem to suggest that BRCA1 could be an important negative regulator of cell cycle that functions through interaction with E2F transcriptional factors and phosphorylation by cyclins/cdk complexes with the zinc ring finger functioning as a major protein-protein interaction domain. If the interactions we observe *in vitro* is also seen *in vivo* then it may be possible that lack or impaired

binding of the disrupted BRCA1 proteins to E2F, cyclins/CDKs in patients with mutations in the zinc finger domain could deprive the cell of an important mechanism for braking cell proliferation leading to the development of breast and ovarian cancers.

**Keywords:** BRCA1a; BRCA1b; zinc finger; cyclins; CDKs; E2F

## Introduction

Mutations in the breast and ovarian cancer susceptibility gene BRCA1, accounts for half of the inherited breast and ovarian cancers (Miki *et al.*, 1994; Easton *et al.*, 1995; Ford *et al.*, 1995) and only 10% of the sporadic ovarian cancers (Futreal *et al.*, 1994; Hosking *et al.*, 1995; Merajver *et al.*, 1995). The BRCA1 cDNA codes for a 1863 amino acid protein with an amino terminal zinc ring finger domain and a carboxy terminal acidic region (Miki *et al.*, 1994) typical of several transcriptional factors. Recently, the C terminal region of BRCA1 was shown to activate transcription in a heterologous GAL-4 system (Chapman and Verma, 1996; Monteiro, 1996; Rao *et al.*, unpublished results). Several groups have cloned and studied the developmental patterns of expression of murine BRCA1 (Lane *et al.*, 1995; Marquis *et al.*, 1995; Abel *et al.*, 1995; Sharan *et al.*, 1995). Expression was found to be high in rapidly proliferating tissues (Lane *et al.*, 1995; Marquis *et al.*, 1995) particularly those undergoing differentiation suggesting a role for BRCA1 in cellular growth and differentiation. We and others have shown the BRCA1 gene product to be a nuclear phosphoprotein (Chen *et al.*, 1995; Rao *et al.*, 1996; Scully *et al.*, 1996), that when over expressed in breast and ovarian cancer cells results in growth inhibition *in vitro* and *in vivo* in breast cancer cells (Holt *et al.*, 1996; Rao *et al.*, unpublished results). Conversely, inhibition of BRCA1 expression by antisense RNA in mouse fibroblasts or by antisense oligonucleotides in breast cancer cells resulted in transformation of mouse fibroblasts as well as increased the rate of growth of breast cancer cells (Thompson *et al.*, 1995; Rao *et al.*, 1996). We have recently reported a new function for the BRCA1 splice variant BRCA1a in the regulation of apoptosis of human breast cancer cells (Shao *et al.*, 1996). Previously, the BRCA1 gene product was shown to be localized in the nucleus (Chen *et al.*, 1995; Rao *et al.*, 1996). Since then there have been several

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differences in the literature regarding the size and subcellular location of BRCA1 (Chen *et al.*, 1995, 1996; Jensen *et al.*, 1996; Scully *et al.*, 1996; Thakur *et al.*, 1997; Wilson *et al.*, 1997). Two proteins BARD1 and Rad51, a human homolog of bacterial Rec A were shown to interact both *in vitro* and *in vivo* with BRCA1 indicating a role for BARD1 in tumor suppression of BRCA1 and a role for BRCA1 in the control of recombination and genomic integrity (Wu *et al.*, 1996; Scully *et al.*, 1977). In the present study, we have further investigated the subcellular localization of BRCA1 protein using several BRCA1 specific antibodies in normal, breast and ovarian cancer cells grown under different serum culture conditions and conclude that the BRCA1 proteins are nuclear phosphoproteins that are transported to the nucleus in the absence of serum. Our results suggest that BRCA1a and BRCA1b, two BRCA1 splice variants that are localized mainly in the cytoplasm with a small fraction going into the nucleus, are phosphoproteins containing phosphotyrosine that associate via their amino-terminal zinc ring finger domain with E2F transcriptional factors, cyclins/cdk complexes suggesting a function for this domain in mediating protein-protein interaction and a role for BRCA1 in cell cycle regulation.

## Results

### *Subcellular localization of BRCA1 proteins*

In an attempt to understand the normal function of the BRCA1 protein, we have generated polyclonal antibodies against different regions of the human BRCA1 protein. Initially, we have studied the subcellular distribution of BRCA1 in normal human mammary epithelial cell Hs578 Bst and several human breast cancer cell lines HBL-100, CAL-51, MDA MB-453, T-47D, BT-474, CAMA-1 and ZR 75-1 by immunohistochemistry and immunofluorescence staining. BRCA1 was detected mainly in the cytoplasm with weak nuclear staining of normal human breast epithelial cell Hs578 Bst and several breast tumor cell lines HBL-100, MDA MB-453, T-47D, BT474, CAMA-1 and ZR 75-1 (Figure 1a). One cell line CAL-51, which was originally obtained from a patient with invasive adenocarcinoma with extensive intraductal involvement (Gioanni *et al.*, 1990), contained three distinct populations of cells, some in which BRCA1 was localized in the cytoplasm, some in the perinucleus and some in the nucleus (Figure 1a). We next studied the subcellular distribution of BRCA1 in two ovarian carcinoma cell lines NIH:OVCAR-3 and SK-OV-3. In NIH:OVCAR-3 cells BRCA1 was localized mainly to the cytoplasm (Figure 1b) and in SK-OV-3 BRCA1 was localized mainly in the nucleus (Figure 1b). We also studied the subcellular distribution of BRCA1 in several normal and tumor cells. BRCA1 was found to be distributed mainly in the cytoplasm of NIH3T3 mouse fibroblast cells and Saos-2 cells (Figure 1c), both in the cytoplasm and nucleus of BRCA1a transfected NIH3T3 cells, HeLa, Colo 320, A431 and PC12 cells (Figure 1c) and mainly in the nucleus with weak cytoplasmic staining of BALB/3T3 cells (Figure 1c). All these results suggested variable, subcellular

distribution of BRCA1 proteins. These results were obtained using different BRCA1 antibodies.

### *Subcellular localization and tyrosine-phosphorylation of BRCA1a and BRCA1b proteins*

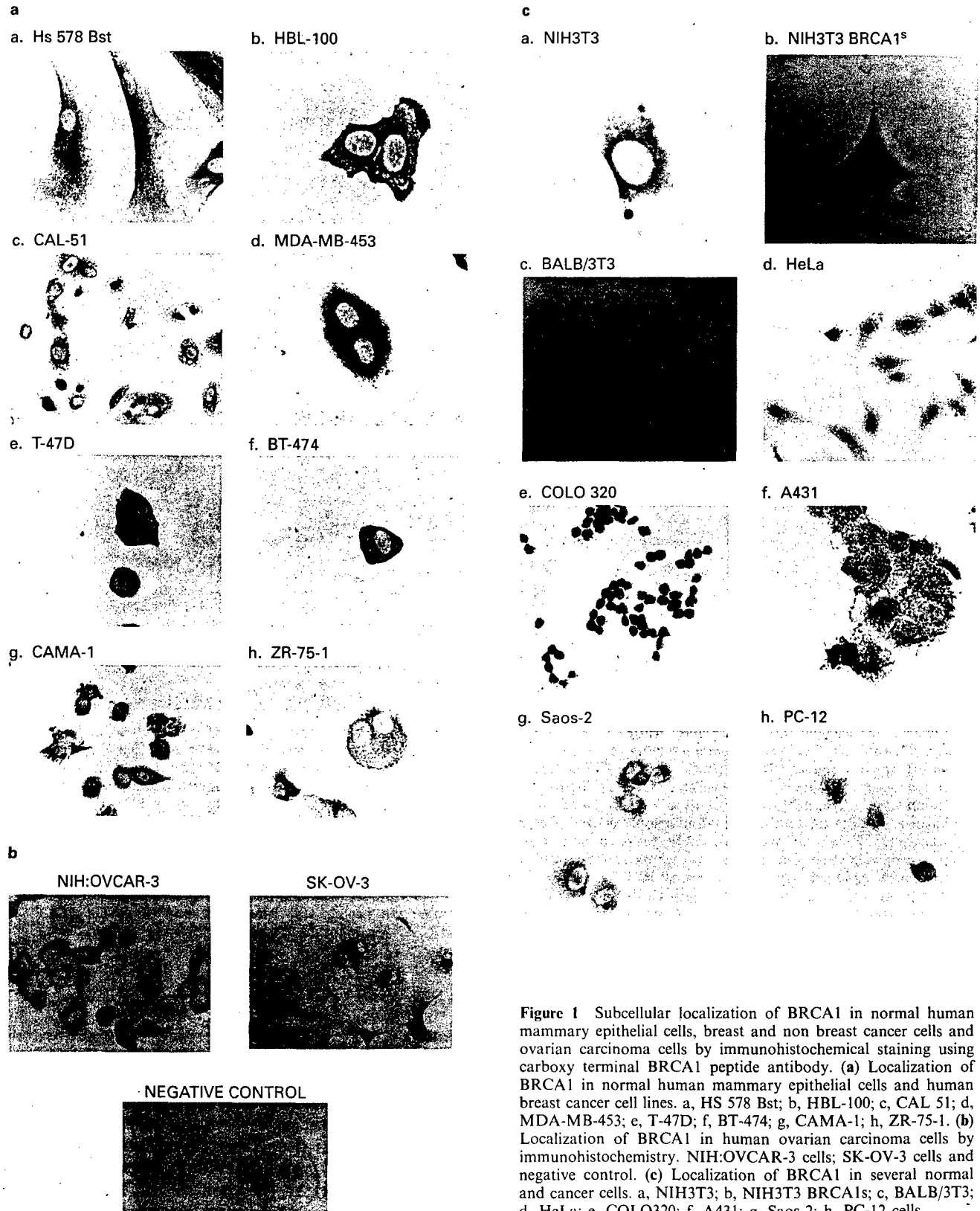
We have next studied the *in vivo* phosphorylation of BRCA1 proteins in HL60 cells. Immunoprecipitation of lysates from HL60 cells labeled with  $^{32}\text{P}$ -phosphoric acid using BRCA1 polyclonal antibody revealed three major bands with molecular weights of  $\approx 175$ , 125 and 110 kD (Figure 2a). All these results suggest BRCA1 to be a phosphoprotein. In order to explain the differences in the subcellular localization of BRCA1, we speculated whether this could be due to the presence of multiple splice variants wherein some could be preferentially sequestered in the cytoplasm and some could translocate to the nucleus as observed recently by other investigators (Thakur *et al.*, 1977; Wilson *et al.*, 1977). So, we subcloned the differentially spliced human BRCA1 cDNA, BRCA1a into pFLAG-CMV-2 expression vector (Eastman Kodak Company) which contains a FLAG epitope-tag sequence at the N-terminal and this tag allows detection of BRCA1a protein with the use of FLAG antibody. When transfected into COS cells the FLAG BRCA1a protein was found to be localized mainly in the cytoplasm with weak nuclear staining (Figure 2b). Subcellular fractionation of the transfected COS cells into total and nuclear fractions followed by Western blot analysis using the same FLAG antibody revealed two polypeptides migrating at 105–110 kD mainly in the cytoplasm with a small fraction of the total BRCA1a protein in the nuclear fractions, which agrees with our immunofluorescence data. These results suggest BRCA1a p110 to be a localized both in the cytoplasm and nucleus, the doublet protein bands observed could represent hyper and hypophosphorylated forms of BRCA1a proteins, similar to the 220 kDa BRCA1 protein (Chen *et al.*, 1996a). Western blot analysis of FLAG immunoprecipitates obtained from p-FLAG-CMV-2-BRCA1a transfected COS cells using phosphotyrosine antibodies revealed BRCA1a to be a phosphoprotein containing tyrosine (Figure 2c, lane 3) which migrated with mobility similar to an endogenous BRCA1 tyrosine phosphorylated band seen in HL60 cells (Figure 2c). All these results suggest that BRCA1a is an  $\approx 110$  kD phosphoprotein which contains tyrosine. Similarly, BRCA1b was also found to be a phosphoprotein phosphorylated on tyrosine migrating with a molecular weight of  $\approx 100$  kD (data not shown). We next studied the subcellular distribution of FLAG-BRCA1a in breast cancer cell line CAL-51 after transfection of pFLAG-CMV-2-BRCA1a plasmid into these cells followed by immunofluorescence analysis. Here, we observed BRCA1a protein to be distributed both in the cytoplasm as well as in the nucleus of serum fed cells, the cytoplasmic staining appeared to be much stronger compared to the weak nuclear staining (Figure 2d).

### *Intracellular localization of BRCA1 proteins in serum-grown and serum deprived mouse fibroblasts*

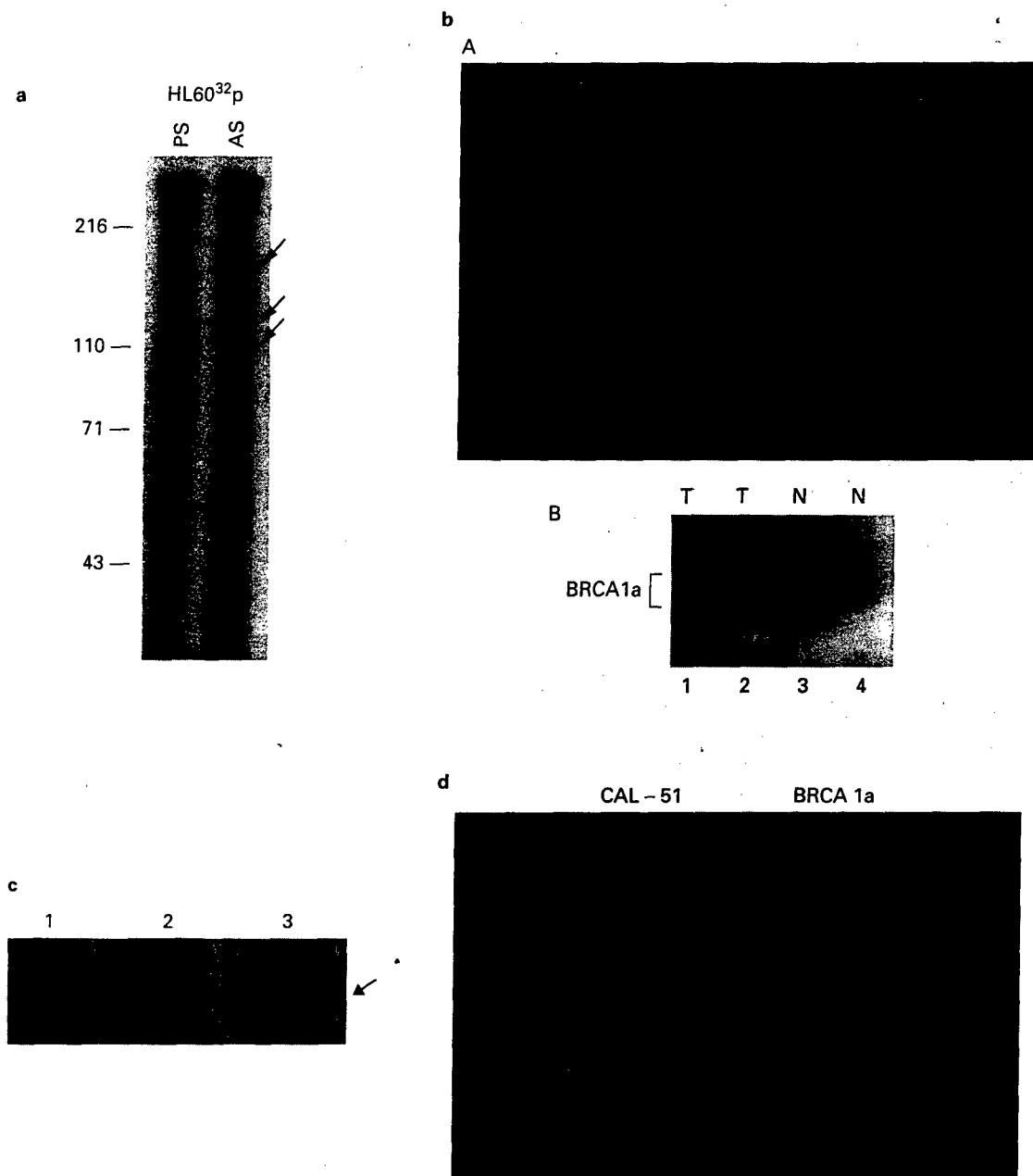
Our results suggested that the interesting differences in the subcellular localization of BRCA1 observed by us

, and others may not be due to using different BRCA1 antibodies or aberrant localization of BRCA1 in breast tumor cells. We therefore speculated whether this could be due to culturing the cells in different serum culture conditions. In order to test the hypothesis whether the nuclear localization of BRCA1 is dependent on the proliferation state of the cell, we studied the subcellular distribution of BRCA1 in asynchronous and serum

deprived NIH3T3 cells using different BRCA1-specific antibodies developed by us by immunofluorescence and immunohistochemical methods. In asynchronous serum fed NIH3T3 cells BRCA1 was found to be localized predominantly in the cytoplasm with weak nuclear staining (Figure 3a). In contrast, in serum starved quiescent cells most of the BRCA1 was found to be localized in the nucleus with little cytoplasmic staining.



**Figure 1** Subcellular localization of BRCA1 in normal human mammary epithelial cells, breast and non breast cancer cells and ovarian carcinoma cells by immunohistochemical staining using carboxy terminal BRCA1 peptide antibody. (a) Localization of BRCA1 in normal human mammary epithelial cells and human breast cancer cell lines. a, HS 578 Bst; b, HBL-100; c, CAL 51; d, MDA-MB-453; e, T-47D; f, BT-474; g, CAMA-1; h, ZR-75-1. (b) Localization of BRCA1 in human ovarian carcinoma cells by immunohistochemistry. NIH:OVCAR-3 cells; SK-OV-3 cells and negative control. (c) Localization of BRCA1 in several normal and cancer cells. a, NIH3T3; b, NIH3T3 BRCA1s; c, BALB/3T3; d, HeLa; e, COLO320; f, A431; g, Saos-2; h, PC-12 cells



**Figure 2** Subcellular localization and tyrosine phosphorylation of endogenous BRCA1 and FLAG-BRCA1a proteins in HL60, CAL-51 cells and COS cells by immunofluorescence and Western blot analysis. (a) Immunoprecipitation of  $^{32}\text{P}$ -labeled HL60 cells with carboxy terminal BRCA1 peptide antibody. The three arrows represent the phosphorylated BRCA1 protein bands. (b) A, Immunostaining of COS cells transfected with FLAG-BRCA1a with FLAG antibody showing mostly cytoplasmic and weak nuclear staining; B, Western blot analysis of COS cells transfected with FLAG-BRCA1a with FLAG antibody shows BRCA1a to be localized both in the total (nucleus and cytoplasm) and nuclear fractions of COS cells. (c) Western blot analysis of FLAG-BRCA1a transfected COS cells shows BRCA1a to be a phosphoprotein containing phosphotyrosine. Lane 1 represents HL60 cells immunoprecipitated with pre-immune serum and Western blotted using phosphotyrosine antibody; lane 2 represents HL60 cells immunoprecipitated with carboxyterminal BRCA1 peptide antibody and then Western blotted using phosphotyrosine antibody; lane 3 represents COS cells transfected with CMV2 FLAG-BRCA1a, immunoprecipitated with FLAG antibody and Western blotted using phosphotyrosine antibody. The arrow represents the 110 kD BRCA1a protein. (d) Immunostaining of CAL-51 cells transfected with CMV2-FLAG-BRCA1a using FLAG antibody, shows BRCA1a to be localized mainly in the cytoplasm with weak nuclear staining

On prolonged incubation in serum free media for 48–72 h the staining was mostly nuclear with typical nuclear dot like pattern (data not shown). When the cells were refed with 10% serum for 24 h the BRCA1 protein was found to relocate back to the cytoplasm with weak nuclear staining similar to the situation seen in asynchronous serum fed NIH3T3 cells (Figure 3a). The same results were obtained using four different antibodies specific to different regions of BRCA1

protein, (data not given) thus ruling out the possibility that nuclear BRCA1 immunofluorescence might be an artifact due to starvation. Simultaneously, we have also determined the DNA content of these cells in the presence and absence of serum by FACS analysis to give a measure of the relative percentage of G1, S, G2/M cells in each sample. All these results seem to suggest that nuclear transport of BRCA1 is not cell cycle dependent and irrespective of the cell cycle state,

BRCA1 protein accumulates in the nucleus in the absence of serum and in the cytoplasm in the presence of serum, in NIH3T3 cells. We have also arrested the cells in G1 phase of the cell cycle by using drugs such as aphidicolin, mimosine and double thymidine block and studied the subcellular localization of BRCA1 protein. Our results suggest that the redistribution of the BRCA1 proteins to the nucleus in drug-treated cells is not as dramatic as seen with serum starved cells (Shao and Rao, unpublished results). It may be possible that accumulation of BRCA1 proteins in the nucleus of serum depleted NIH3T3 cells may be responsible for the induction of apoptosis seen previously in BRCA1a transfected NIH3T3 cells (Shao *et al.*, 1996). Two splice variants BRCA1  $\Delta$ 672-4095 (Thakur *et al.*, 1997) and BRCA1  $\Delta$ 11b which codes for a 110 kD protein (Wilson *et al.*, 1997) were both shown to be localized to the cytoplasm by immunostaining. Surprisingly, the authors found substantial levels of BRCA1  $\Delta$ 11b to be in the nuclear fraction on immunoblotting (Wilson *et al.*, 1997), similar to what we have observed with BRCA1a and BRCA1b in COS cells. The molecular weight of the p110 kDa BRCA1a polypeptide reported in this and earlier studies (Shao *et al.*, 1996) is comparable to the 110 kDa BRCA1  $\Delta$ 11b. Thus even though the reported nuclear localization signal (Thakur *et al.*, 1997; Wilson *et al.*, 1997) for BRCA1 is missing in BRCA1a and BRCA1b proteins, some of which still gets transported to the nucleus suggesting that both BRCA1a and BRCA1b may carry potential nuclear localization signals. It may be possible that there can be a serum-responsive regulatory protein that could be responsible for retention of BRCA1 protein within the cytoplasm of serum fed cells similar to NF- $\kappa$ B (Baeuerle and Baltimore, 1988) and the levels of which could determine the subcellular localization of BRCA1 protein. We are presently investigating these possibilities.

*Subcellular localization of BRCA1 proteins in breast and ovarian cancer cells grown in the presence and absence of serum*

Since all these experiments were done in mouse fibroblasts, which may not be physiologically relevant to BRCA1 function we studied the subcellular distribution of BRCA1 in asynchronous and growth arrested serum deprived normal human breast epithelial cells Hs578 Bst, breast cancer cells HBL-100, ZR-75-1, CAMA-1 and ovarian carcinoma cells NHOVCA-3, using different BRCA1 specific polyclonal antibodies by indirect immunofluorescence and immunohistochemical methods. We have used only those cell lines in which BRCA1 was found to be localized to the cytoplasm in asynchronous conditions. Our results suggest BRCA1 to be localized mainly in the cytoplasm of serum fed asynchronous normal breast epithelial cells Hs578 Bst, breast tumor cells HBL-100, ZR-75-1, CAMA-1 and ovarian carcinoma cell NHOVCA-3 (Figure 3b-f) and predominantly in the nucleus of growth arrested serum deprived Hs578 Bst, HBL-100, ZR-75-1 CAMA-1 and ovarian carcinoma cell line NHOVCA-3 (Figure 3b-f). All these results suggest that the nuclear or cytoplasmic transport of BRCA1 is not spontaneous but is

controlled by the extracellular environment with serum growth factors inhibiting the nuclear transport of the BRCA1 protein. It remains to be seen whether posttranslational modification (such as phosphorylation, etc.) of BRCA1 proteins plays a role in the subcellular localization of these proteins. The scenario we observe with BRCA1 is different to that seen for the c-Fos protein whose nuclear translocation appears to depend on the continuous stimulation of cells by serum factors (Roux *et al.*, 1990).

*Detection of cellular proteins that interact with GST-BRCA1 fusion protein*

Since the subcellular localization of BRCA1 is dependent on the presence of serum factors, and to investigate whether BRCA1 protein-protein interactions are of biological significance in the growth inhibitory, tumor suppressor and death inducing functions of BRCA1 protein, we studied the interaction of BRCA1 with cellular proteins. The amino terminal region of BRCA1 contains a zinc ring finger domain (Miki *et al.*, 1994) which are known to interact with DNA/RNA either through direct binding or indirectly by mediating protein-protein interactions. We have expressed a fusion protein that contains GST and the zinc finger domain of BRCA1 (residues 1-76) in bacteria using the Gex 2T expression vector system. In order to detect cellular proteins that interact with GST-BRCA1 fusion protein, whole cell lysates of human breast cancer cells ZR-75-1 or CA1-51 metabolically labeled with  $^{35}$ S-methionine were incubated with either GST or the GST-BRCA1 fusion protein immobilized on glutathione-agarose beads (GSH-beads). The beads were washed, lysed in SDS sample buffer and subjected to SDS-PAGE. SDS-PAGE analysis of the bound complex revealed bands with relative molecular weight of  $\approx$ 32 kD and  $\approx$ 65 kD which bound exclusively to the GST-BRCA1 fusion protein and not to GST (Figure 4a). These bands were consistently detected in ZR-75-1, CA1-51 and HL 60 cell lysates and were designated as p65 BIP and p32 BIP respectively (data not shown).

*Direct association of p65 BIP and p32 BIP with BRCA1*

To determine whether p65 BIP and p32 BIP directly interact with BRCA1, we performed a far Western blot analysis. The BIP complexes prepared from CA1-51 cell extract were separated on SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with  $^{32}$ P-labeled GST-TK-BRCA1 fusion protein. Since GST-TK-BRCA1 contains consensus phosphorylation site for protein kinase C at the amino terminal end, the purified protein can be  $^{32}$ P-labeled by an *in vitro* protein kinase reaction. GST-BRCA1 bound to both p65 BIP and p32 BIP unlike GST protein (Figure 4b). These results suggest that both p65 BIP and p32 BIP bind strongly to BRCA1 even under stringent conditions without any mediating proteins (Figure 4b).

*Expression of BRCA1 binding proteins in different cell lines*

To determine the distribution of BRCA1-binding protein in various cell lines, we metabolically labeled

promyelocytic cell line HL60, breast cancer cell lines ZR-75-1 and CAL-51 cells and performed the GST-pull down assay as described previously. Both p65 BIP

and p32 BIP were detected in all cell lysates examined (data not given) although at variable levels suggesting the ubiquitous expression of p65 BIP and p32 BIP.

a. NIH3T3 10% FBS



b. NIH3T3 serum free 48h



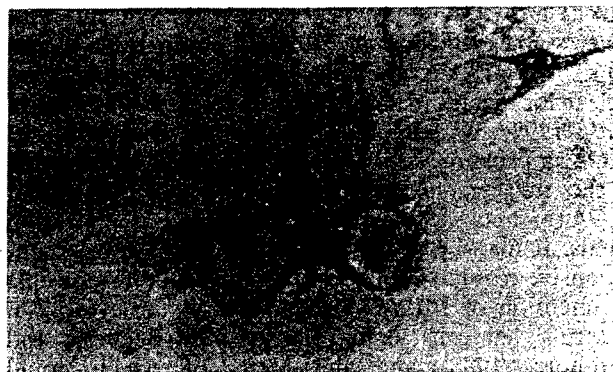
c. NIH3T3 stimulated 24h



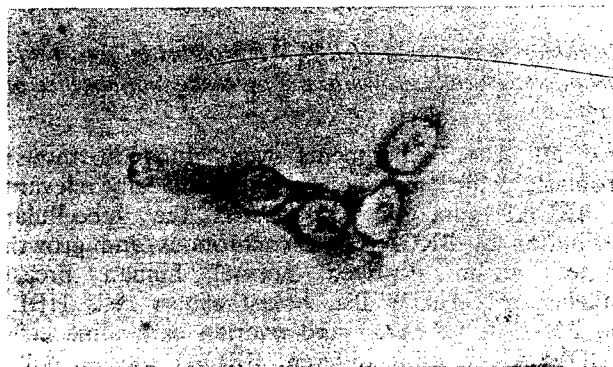
c. 10% FBS



b. serum free 48h



c. serum free 72h



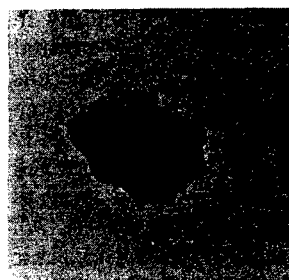
a. HS 578 Bst 10%FBS



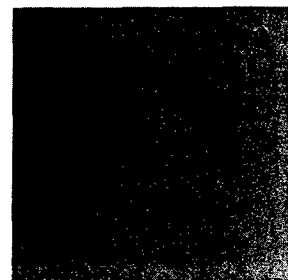
b. HS 578 Bst serum free 48h



a. ZR-75-1 10% FBS



b. ZR-75-1 serum free

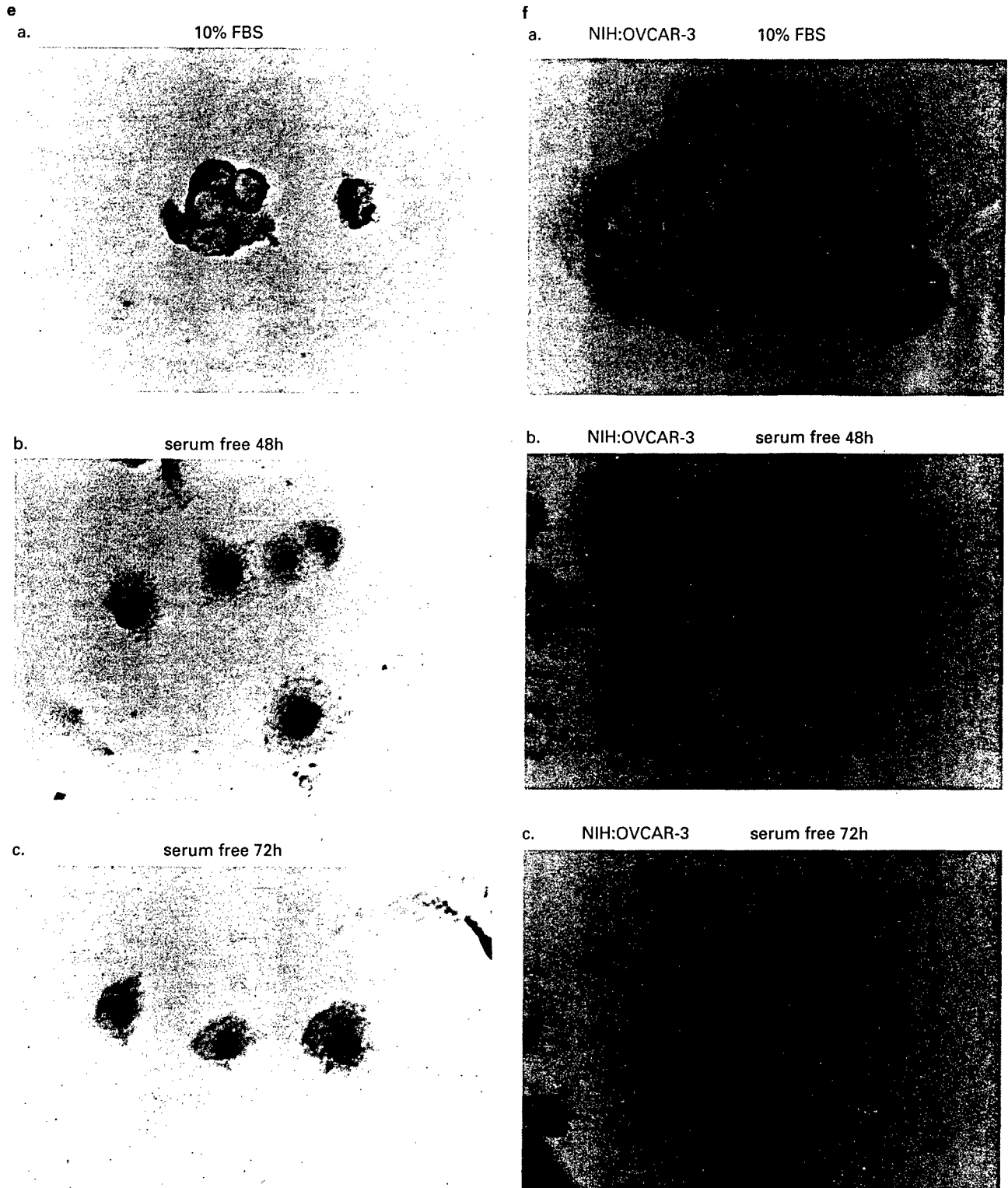




# *Association of BRCA1 proteins with E2F, cyclins/CDKs*

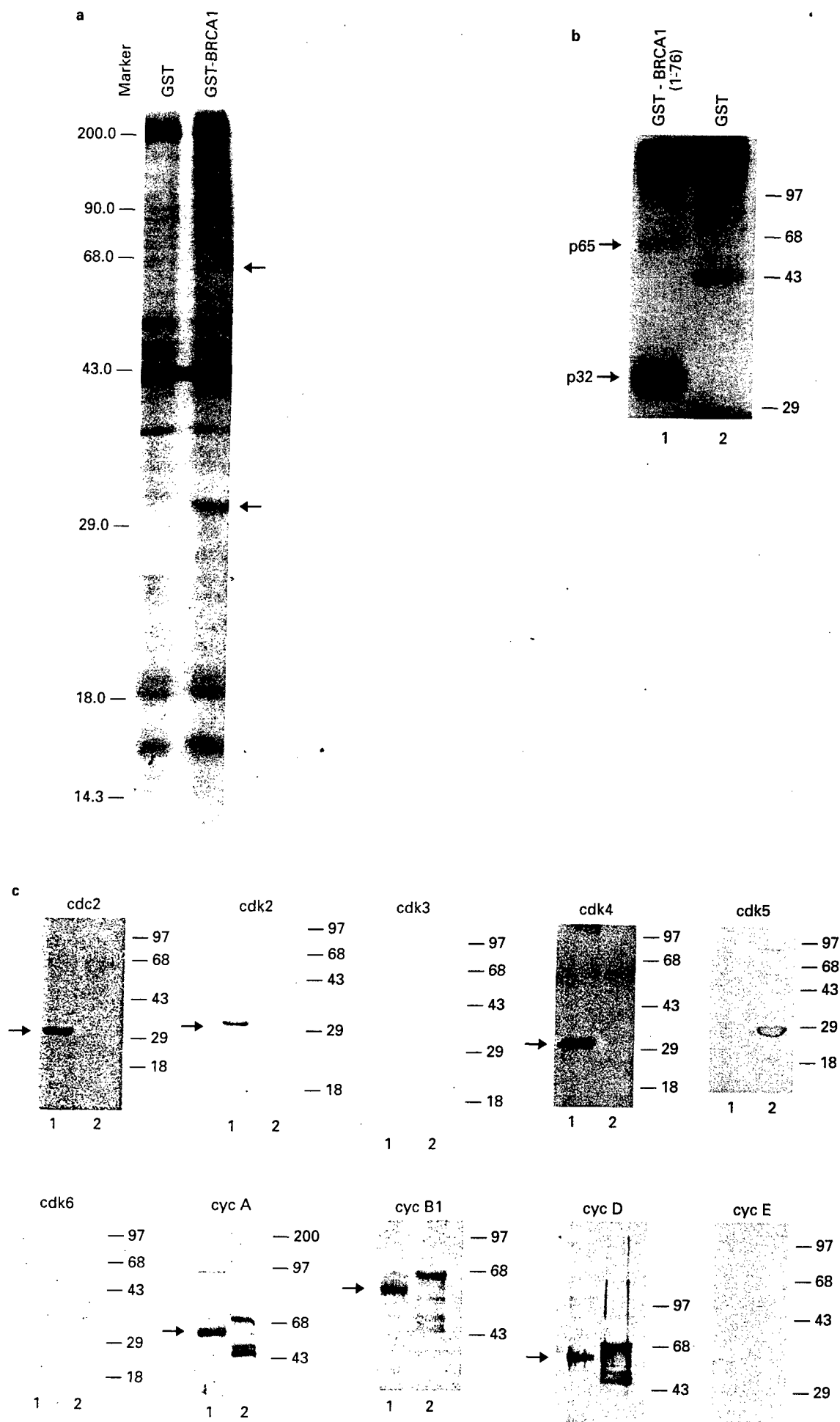
Recently, cdk-2 and other kinases associated with cyclins D and A were shown to phosphorylate

BRCA1, suggesting a role for cdk's in regulating the activity of BRCA1 proteins (Chen, 1996a) and the growth inhibitory function of BRCA1 also suggested a role in cell cycle control (Holt *et al.*, Rao *et al.*,



**Figure 3** BRCA1 localizes to the nucleus in serum deprived NIH3T3, normal mammary epithelial cells, breast and ovarian cancer cells by immunohistochemical analysis. Intracellular localization of BRCA1 protein in serum-starved and serum fed NIH3T3 cells. (a) using amino-terminal BRCA1 peptide antibody. The above results were also confirmed using carboxy terminal BRCA1 peptide antibody, and an antibody raised against recombinant BRCA1 protein (aa 1-331), ethyl green was used as a positive control for nuclear staining (data not given). (b) BRCA1 redistributed to the nucleus in serum deprived normal human mammary epithelial cells HS578 Bst using immunohistochemical analysis. (c) BRCA1 translocated to the nucleus in serum deprived human breast cancer cells HBL-100. (d) ZR-75-1 cells, and (e) CAMA-1 cells using immunohistochemical analysis. (f) BRCA1 was found to translocate to the nucleus of serum deprived human ovarian carcinoma cell line NIH:OVCA-3. The above results were repeated using three different BRCA1 specific antibodies



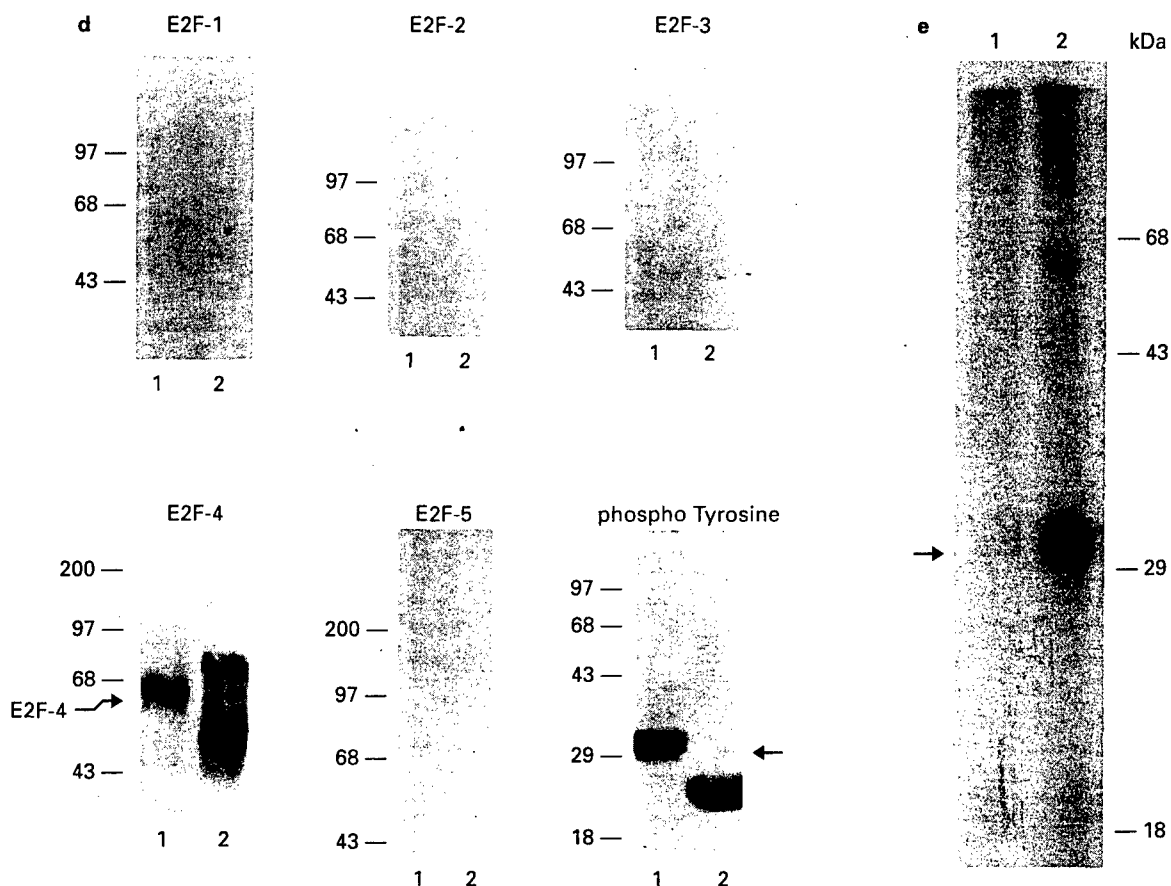


unpublished results). Since tumor suppressor genes like Rb regulate cell cycle by interaction with transcription factor E2F, cyclins/cdk's (Lees *et al.*, 1992; Kato *et al.*, 1993; Ewen *et al.*, 1993; Weinberg, 1995) and the sizes of BIPs are close to E2F, cyclins/CDKs, we speculated whether BRCA1 could similarly associate with E2F, cyclins/CDKs. We therefore incubated cell lysates obtained from CAL-51 cells with GST-immobilized on GSH-beads and BRCA1 fusion protein conjugated GSH-beads. The beads were then washed and heated in SDS sample buffer. The BIP complexes were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membrane and probed with antibodies specific to cdc2, cdk2, cdk3, cdk4, cdk5, cdk6, cyclin A, cyclin B1, cyclin D1, cyclin E, E2F-1, E2F-2, E2F-3, E2F-4 and E2F-5 as described previously (Rao *et al.*, 1996). The BIP complexes were recognized by antibodies specific to cdc2, cdk-2, cdk-4, cyclin D1, cyclin A, cyclin B1

(Figure 4c) and E2F-4 (Figure 4d), but not to cdk3, cdk-5, cdk-6, cyclin E, E2F-1, E2F-2, E2F-3 and E2F-5. All these results suggest association of BRCA1 with cyclin A, D1 and B1, cdc2, cdk-2, cdk-4 and E2F-4, but not to cdk-3, cdk-5, cdk-6, cyclin E, E2F-1, E2F-2 and E2F-5.

#### Tyrosine phosphorylation and kinase activity of BRCA1 interacting proteins

We next wanted to examine whether p32 BIP and p65 BIP contain phosphotyrosine. The BIP complexes obtained from CAL-51 cells were immunoblotted with a phosphotyrosine antibody (Figure 4d). Band corresponding to p32 was detected (Figure 4d) indicating that p32 BIP contains phosphotyrosine, a characteristic specific to cyclin associated protein kinases. We have also tested the BRCA1 immunoprecipitates from CAL-51 cells for kinase activity. Our



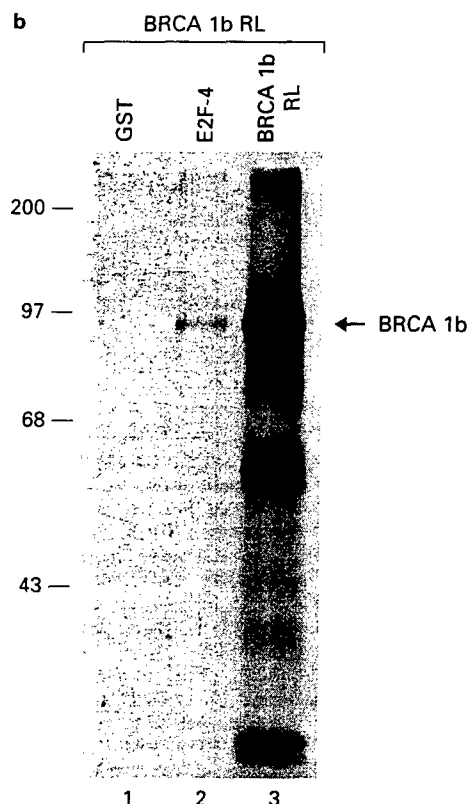
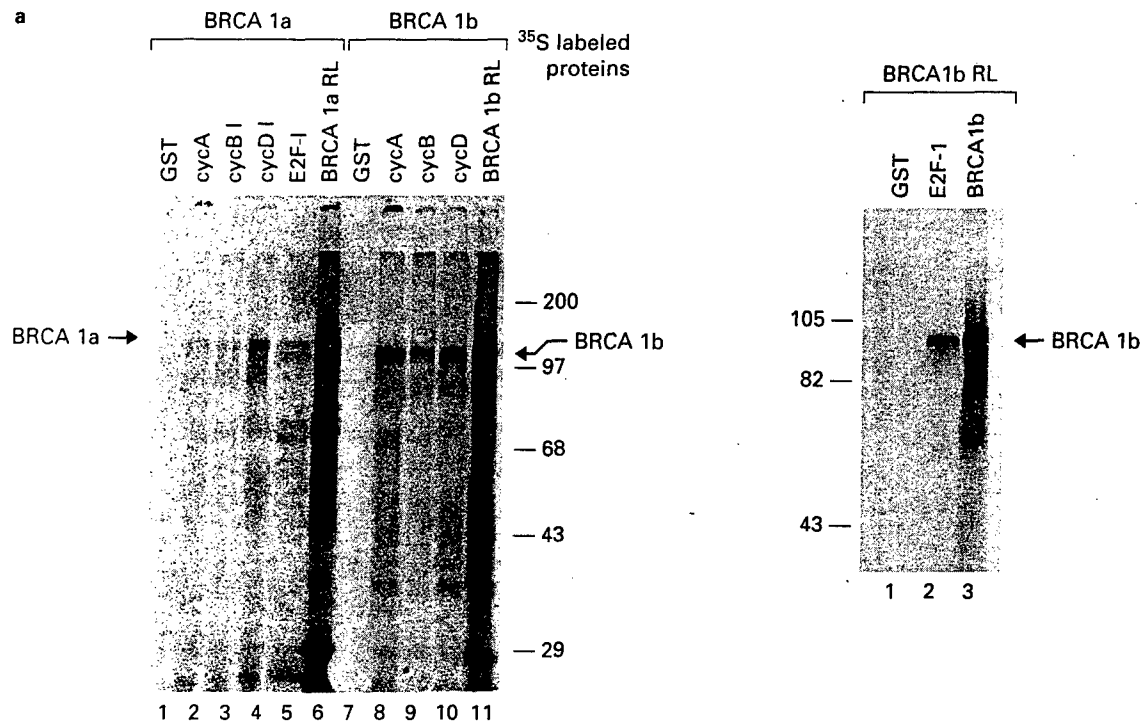
**Figure 4** Identification of BRCA1 interacting proteins (BIP) that associate with E2F's cyclins/CDKs in breast cancer cells. (a) Detection of BRCA1-binding proteins. Lysates of <sup>35</sup>S-methionine-labeled CAL-51 cells were incubated with either GST or the GST-BRCA1 fusion protein immobilized on GSH beads. The bound proteins were washed, subjected to 10% SDS-PAGE and visualized by fluorography. The position of the major ≈p32 BIP complex is indicated on the right with a solid arrow and the minor ≈p65 BIP complex is represented by a small arrow on the right. The positions of the <sup>14</sup>C-labeled protein standards and their sizes are shown on the left. (b) Direct binding of BRCA1 with p32 BIP and p65 BIP by far Western blot analysis. CAL-51 cell extracts were passed through GST and GST-BRCA1 immobilized on glutathione beads. The protein complexes were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking, the filters were incubated with the <sup>32</sup>P-labeled GST-BRCA1 fusion protein. The arrows represent the p32 and p65 BIP proteins. (c) Association of BRCA1 with cyclin/cdk's. Unlabeled cell extract from CAL-51 cells were incubated with GST conjugated GSH-BRCA1 beads (lane 1) or with GST-conjugated GSH-beads (lane 2). The beads were then washed and subjected to 10% SDS-PAGE. The proteins were transferred onto nitrocellulose membrane and subjected to Western blot analysis using antibodies specific to cdc2, cdk2, cdk3, cdk4, cdk5, cdk6, cyclin A, cyclin B, cyclin D and cyclin E obtained from Santa Cruz Biotechnology. (d) Association of BRCA1 with transcriptional factor E2F-4 and the p32 BIP complex are tyrosine phosphoproteins. CAL-51 cell extracts were passed through GST-BRCA1 GSH beads (lane 1) or GST-GSH beads (lane 2) and the bound proteins were subjected to SDS-PAGE and Western blot analysis using antibodies specific to E2F-1, E2F-2, E2F-3, E2F-4, E2F-5, and phosphotyrosine obtained from Santa Cruz Biotechnology. The arrows represent E2F-4 and tyrosine phosphorylated p32 BIP proteins respectively. (e) Kinase activity of BRCA1 immunocomplexes on histone H1. Lane 1, buffer control; lane 2, histone H1. The arrow represents phosphorylated histone H1

results demonstrate that these immunocomplexes show histone H1 kinase activity confirming the association of BRCA1 with cyclins/cdk kinase complexes (Figure 4e).

*In vitro interactions of BRCA1a, BRCA1b with cyclins, E2F and CDKs*

To examine the binding of cyclin A, cyclin B1, cyclin D1, E2F-1 and E2F-4 with BRCA1a and BRCA1b *in*

*vitro*, we inserted the cDNA sequences encoding BRCA1a and BRCA1b into pcDNA3 expression vectors (Rao *et al.*, unpublished results). *In vitro* transcription and translation of BRCA1a and BRCA1b in the presence of [<sup>35</sup>S]methionine generated radiolabeled BRCA1a and BRCA1b polypeptides of ~110 and ~100 kD respectively (Figure 5a lane 6 and 11). These radiolabeled proteins were passed through GST-cyclin A, GST-cyclin B1, GST-cyclin D1, GST-

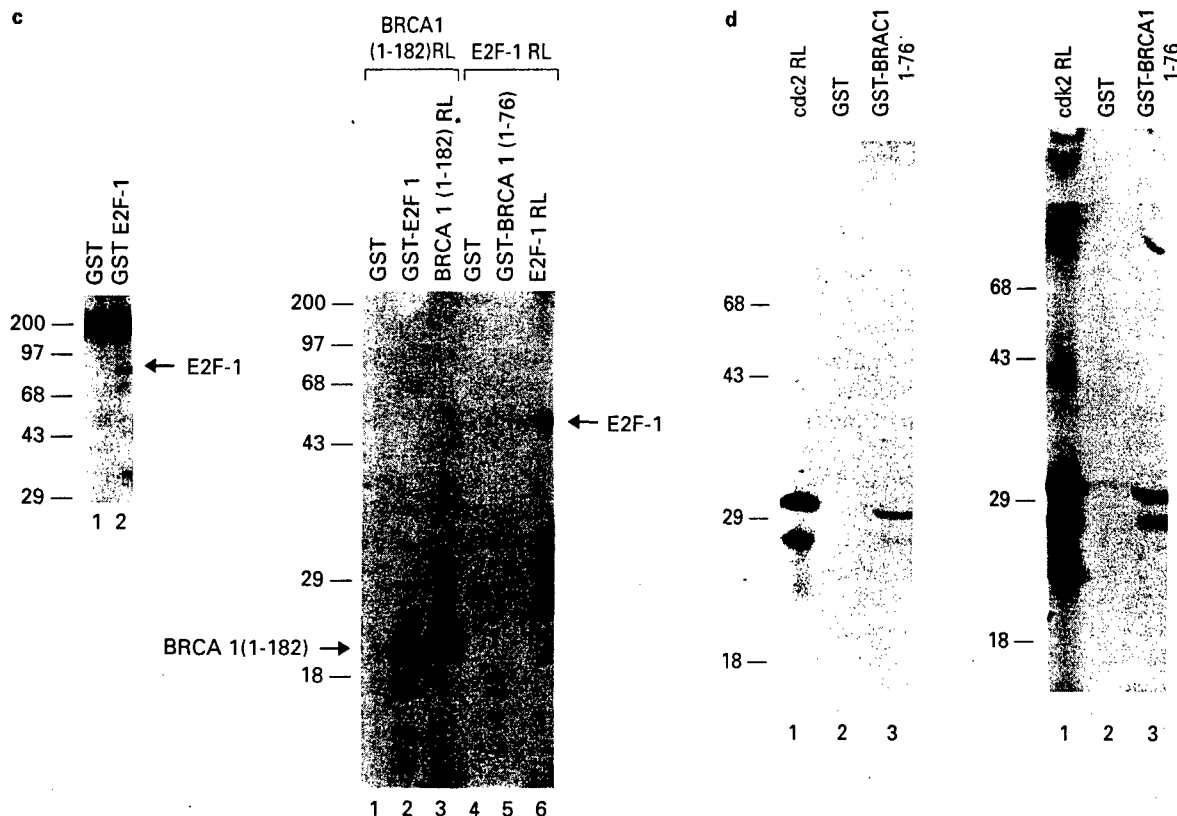


E2F-1, GST-E2F-4 and GST respectively. Both *in vitro* translated BRCA1a and BRCA1b specifically bind to GST-cyclin A, GST-cyclin B1, GST-cyclin D1, GST-E2F-1 and GST E2F-4 unlike GST alone (Figure 5a, b). The BRCA1a splice variant bound at a reduced level compared to BRCA1b to all these different proteins. It may be possible that phosphorylation may regulate the binding of BRCA1a and BRCA1b proteins to cyclins, cdk's and E2F transcriptional factors. To confirm our results, further GST and GST E2F-1 fusion proteins were subjected to far Western blot analysis using  $^{32}$ P-labeled amino-terminal BRCA1 (GST-BRCA1a amino acids 1–76, numbering from first ATG codon) fusion protein. The GST E2F-1 fusion protein band hybridized specifically to BRCA1 (Figure 5c). To further confirm the results, a fragment of BRCA1 encoding the amino-terminal 182 amino acids (amino acids 1–182) was *in vitro* translated and assayed for binding to GST-E2F-1 protein. The BRCA1 polypeptides bound specifically to GST-E2F-1 (Figure 5c). In a reciprocal assay, we *in vitro* translated the full length human E2F-1 cDNA and assayed it for binding to GST-BRCA1 zinc finger fusion protein (amino acids 1–76). The E2F-1 polypeptide bound very weakly to GST BRCA1 (Figure 5c). These results suggest that the amino-terminal 76 amino acids of BRCA1 were sufficient to

provide specific association with E2F-1. The amount of E2F-1 and E2F-4 that got bound to BRCA1 appeared to be relatively low. It may be possible that E2Fs may need to associate with other proteins in order to bind efficiently to BRCA1. Similarly, we have *in vitro* translated human cdc2, cdk2, cdk4 and cdk5. The proteins thus generated were assayed separately for binding to GST and GST-BRCA1 (amino acids 1–76) fusion proteins. GST-BRCA1 specifically bound to cdc2 and cdk2 (Figure 5d) but not to cdk4 and cdk5 (data not given). All these results suggest that BRCA1 zinc finger domain can interact directly with cdc2 and cdk2.

## Discussion

In the present report, we have presented data regarding the subcellular localization; phosphorylation and protein-protein interactions of BRCA1 and two BRCA1 isoforms, BRCA1a and BRCA1b. Initially, we have generated polyclonal antibodies against different regions of the human BRCA1 protein and used them to analyse BRCA1 expression in several normal, breast and ovarian cancer cells grown under different serum culture conditions. Our results suggest that BRCA1 proteins accumulate in the nucleus in the



**Figure 5** Direct physical interaction *in vitro* of BRCA1a and BRCA1b with cyclins, CDKs, E2F-1 and E2F-4 transcriptional factors. (a) *In vitro* translated BRCA1a and BRCA1b bind to GST-cyclin A, GST-Cyclin B1, GST-Cyclin D1, GST-E2F-1, 2  $\mu$ l of the *in vitro* translated BRCA1a and BRCA1b were run as controls. Arrows represent *in vitro* translated BRCA1a (p110) and BRCA1b (p100). (b) *In vitro* translated BRCA1b binds to GST-E2F-4. Arrow represents *in vitro* translated BRCA1b (p100). (c) The panel on the left represents far Western blot analysis of BRCA1 (amino acids 1–76) binding directly to E2F-1. The arrow represents the position of GST-E2F-1 fusion protein. The panel on the right represents *in vitro* translated BRCA1 (amino acids 1–182) and *in vitro* translated full length E2F-1 binding to GST-E2F1 and GST-BRCA1 (amino acids 1–76) respectively. The arrows represent *in vitro* translated BRCA1 (amino acids 1–182) and E2F-1. (d) *In vitro* translated full length cdc2 and cdk2 bind to GST-BRCA1 (amino acids 1–76). 2  $\mu$ l of *in vitro* translated cdc2 (left panel) and cdk2 (right panel) were loaded as controls

absence of serum and in the cytoplasm in the presence of serum in NIH3T3 cells and some breast and ovarian cancer cells. It may be possible that the nuclear or cytoplasmic transport of BRCA1 is not spontaneous but is controlled by the extracellular environment with serum inhibiting the nuclear transport of the BRCA1 protein. Alternatively, it may be possible that a protein factor present in the cytoplasm of serum fed cells could be responsible for retention of BRCA1 protein within the cytoplasm, similar to NF-KB (Baeuerle and Baltimore, 1988) and the levels of which could determine the subcellular localization of BRCA1 protein. We are presently investigating these possibilities.

The BRCA1 cDNA codes for a 1863 amino acid protein (Miki *et al.*, 1994) with an amino terminal zinc ring finger motif and two putative nuclear localization signals, suggesting that it might be a nuclear protein (Miki *et al.*, 1994; Lane *et al.*, 1995). There have been several discrepancies in the literature regarding the size and subcellular localization of BRCA1 (Chen *et al.*, 1995, 1996; Rao *et al.*, 1996; Wilson *et al.*, 1997; Scully *et al.*, 1996; Jensen *et al.*, 1996). Previously, we have reported BRCA1 proteins to be localized mainly in the nucleus and some in the cytoplasm of Rat-1 and MCF7 cells (Rao *et al.*, 1996). Subsequently, we have isolated two new alternately spliced BRCA1 transcripts referred to as BRCA1a (p110) and BRCA1b (p100) (Rao *et al.*, unpublished results) and developed mouse fibroblast cell lines and human breast cancer cell lines expressing BRCA1a proteins (Shao *et al.*, 1996). Our results indicate that over expression of BRCA1a induces apoptosis in NIH3T3 and MCF-7 cells after calcium ionophore treatment indicating that BRCA1 genes may play a critical role in the regulation of apoptosis (Shao *et al.*, 1996). In this report, we have studied the subcellular localization of BRCA1a and BRCA1b proteins using FLAG epitope tagged CMV promoter vectors. Our data using immunofluorescence and immunoblotting analysis indicate cytoplasmic and nuclear localization of FLAG BRCA1a and FLAG BRCA1b proteins. Recently, two other groups (Thakur *et al.*, 1997; Wilson *et al.*, 1997) have identified two BRCA1 splice variants BRCA1 $\Delta$ 672-4092 (which lacks exon 11) and BRCA1 $\Delta$ 11b (which lacks majority of exon 11) both of which were found to localize to the cytoplasm by immunostaining. These investigators have mapped a nuclear localization signal in exon 11 of BRCA1 which is missing in BRCA1 $\Delta$ 672-4095 and BRCA1 $\Delta$ 11b suggesting that splicing may regulate the function of BRCA1 by altering the subcellular localization of these proteins (Thakur *et al.*, 1997; Wilson *et al.*, 1997). Interestingly, BRCA1 $\Delta$ 11b was also found to be present in significant quantities in the nuclear fractions on immunoblotting analysis (Wilson *et al.*, 1997), similar to what we have observed with BRCA1a and BRCA1b proteins (this study). We have also found the BRCA1 proteins to accumulate in the cytoplasm in the presence of serum and in the nucleus in the absence of serum. All these results suggest that the nuclear localization of BRCA1 may be regulated by external stimuli, phosphorylation or protein-protein interactions. BRCA1 expression was shown to be high in tissues that are undergoing rapid growth and differentiation (Lane *et al.*, 1995; Marquis *et al.*,

1995). Recently the BRCA1 protein was also shown to be expressed and phosphorylated in a cell cycle dependent manner (Chen *et al.*, 1996). The BRCA1 m-RNA levels were found to be high in exponentially growing cells and in cells just prior to entry into S-phase but decreased upon growth factor withdrawal or after treatment with transforming growth factor  $\beta$ -1 (Gudas *et al.*, 1996) suggesting cell cycle regulation of BRCA1 expression. In an attempt to isolate proteins that interact with BRCA1, we have detected two cellular proteins (p65 BIP, p32 BIP) that specifically interact with BRCA1. Western blot analysis of BIP indicated association with E2F, cyclins and CDKs and *in vitro* translated BRCA1a and BRCA1b proteins interacted directly with transcription factor E2F, cyclins and CDK's suggesting a role for these proteins in regulating the biological activity of BRCA1 proteins.

In summary our results indicate BRCA1 gene products to be nuclear tyrosine phosphoproteins (Rao *et al.*, 1996; Chen *et al.*, 1995; this study) that translocate to the nucleus in the absence of serum, function as tumor/growth suppressors (Rao *et al.*, 1996; Rao *et al.*, unpublished results); Holt *et al.*, 1996), inducers of apoptosis (Shao *et al.*, 1996) and associate *in vitro* with E2F transcriptional factors, cyclins and cdk complex, suggesting a role for CDKs in regulating the biological activity of BRCA1. Several critical growth regulators like the product of the pRB, a tumor suppressor protein have been shown to associate with E2F both *in vivo* and *in vitro* (Weinberg, 1995) resulting in net inhibition of E2F-mediated transactivation and E2F release from pRB is an important event in the activation of genes required for S-phase entry (Weinberg, 1995). It may be possible that tumor suppressor proteins like BRCA1 may similarly interact with E2Fs and regulate cell proliferation. The interaction of BRCA1 zinc ring finger domain to E2Fs, cyclins/cdk complexes assigns a function for this domain in mediating protein-protein interaction. The function of BRCA1 zinc ring domain becomes important since it is the location of some of the most frequently occurring mutations linked to breast and ovarian cancers. One of the BRCA1 mutations contains a frame shift in exon 2 (188 del 111; Miki *et al.*, 1994) which removes the zinc finger domain from the protein. The 185 del AG mutation, which is the most common BRCA1 mutation seen to date that occurs 1 in 100 Ashkenazi Jewish individuals (Shattuck-Eiden *et al.*, 1995) disrupts the BRCA1 gene product at the first residue of the C<sub>3</sub>HC<sub>4</sub> domain. These frequent missense mutations Cys 61, Gly, Cys 64, Gly, and Cys 64 Tyr also disrupt the zinc finger domain structure. It may be possible that lack or impaired binding of the disrupted BRCA1 protein to E2F, cyclins/CDKs in patients with mutations in the zinc ring domain could deprive the cell of an important mechanism for regulating cell proliferation leading to the development of breast cancer. Future efforts will be directed towards showing the identity of p32 BIP and p65 BIP and identifying the cyclins/CDKs and E2Fs family members which specifically interact with BRCA1 *in vivo*. It remains to be seen whether the interactions we observe *in vitro* can be seen *in vivo* and if they are physiologically relevant.

## Materials and methods

### Cell lines

NIH3T3 cells and their derivatives, MCF-7, MDA-MB-453 and A431 cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (PS); HS578 Bst cells were grown in DMEM supplemented with 10% FBS, 1% PS and 30 ng/ml EGF; CAL-51 cells were grown in DMEM containing 10% FBS, 1% PS, 0.6 µg/ml bovine insulin,  $5 \times 10^{-3}$  µg/ml transferrin and 146 mg/Liter glutamine; ZR 75-1 and COLO 320 were grown in RPMI 1640 supplemented with 10% FBS, 1% PS and 10 µg/ml bovine insulin; NIH:OVCA-3 cells were cultured in RPMI 1640 supplemented with 20% FBS, 1% PS and 10 µg/ml bovine insulin; PC12 cells were grown in RPMI 1640 supplemented with 10% horse serum and 5% FBS, 1% PS; SK-OV-3 and HBL 100 cells were grown in McCoy's 5a medium supplemented with 10% FBS and 1% PS; Saos-2 cells were grown in McCoy's 5a medium supplemented with 15% FBS and 1% PS; CAMA-1 cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% FBS and 1% PS. All the cell lines except CAL-51 cells were obtained from American type culture collection (Rockville, MD).

### Immunohistochemistry

The different cell lines in the logarithmic stage of growth cultured in chamber slides were made quiescent in the presence of DMEM only for a period of 24–72 h. Cells were then washed in PBS and subjected to immunohistochemistry analysis using rabbit anti BRCA1 carboxy-terminal or an amino terminal peptide antibody (Santa Cruz Biotechnology Inc.), rabbit polyclonal antibody generated against GST fusion proteins containing amino acids 1 to 76 and amino acids 1 to 331 of the BRCA1 protein as described previously (Rao et al., 1996).

### Plasmid construction and transfections

BRCA1a cDNA and BRCA1b cDNA (Rao, unpublished results) were subcloned into pFLAG-CMV vector (Eastman Kodak Company) by PCR. Purified DNA 5 µg for chamber slides and 20 µg for 100 mm petri dishes of pFLAG-CMV-2 expression vector or pFLAG-CMV-2 expression vector containing the BRCA1a cDNA were transfected into COS and CAL-51 cells using the Invitrogen kit according to the manufacturer's recommendations. After 48–72 h post transfection cells were processed for immunofluorescence analysis or the cell extracts were subjected to Western blot analysis using FLAG M2 antibody (Eastman Kodak Company) as described previously (Rao et al., 1996).

### Immunofluorescence

Immunofluorescence analysis was done as described previously (Rao 1996; Shao et al., 1996) except with slight modifications. In brief, pFLAG-CMV-2 BRCA1a transfected COS and CAL-51 cells cultured in chamber slides were fixed in 3.7% formaldehyde at room temperature, followed by washing in PBS and PBS with 0.05% Triton X-100 and blocking in blocking solution (4% normal goat serum, 0.05% Triton X-100 in PBS) for 10 min at room temperature. The cells were incubated with primary anti FLAG M2 antibody diluted 1:100 for COS and CAL-51 cells, then washed three times with PBS, blocked with blocking solution for an additional 10 min at room temperature followed by incubation with the secondary antibody (FITC conjugated goat anti mouse IgG). After rinsing in PBS, the slides were mounted with

mounting media and photographed on a confocal microscope.

### Preparation of total nuclear extract and Western blot analysis

COS cells were harvested 48 h post transfection by washing in PBS and treating with trypsin. For preparing total cell extracts the cells were lysed in RIPA buffer and the lysate was centrifuged at 14 000 r.p.m. for 30 min at 4°C. The supernatant was assayed for protein concentration by Bradford's method (Bio-Rad) and  $\approx 50$ –100 µg of protein was subjected to Western blot analysis as described previously (Rao et al., 1996). The nuclear extract was prepared as described (Hurst et al., 1990). In brief, cells were lysed in nuclear extract buffer I (Hurst et al., 1990), centrifuged at high speed for 1 min at room temperature. The crude nucleus was suspended in nuclear extract buffer II (Hurst et al., 1990). Nuclear debris was removed by centrifugation for 1 min at room temperature. The supernatant was diluted by the addition of 20 mM HEPES (pH 7.4). The protein concentrations were determined by Bradford's method (Bio-Rad) and  $\approx 50$ –100 µg of protein was subjected to Western blot analysis.

For Western blotting analysis  $\approx 50$ –100 µg of cell/nuclear extract in SDS sample buffer were loaded on a 10% SDS-PAGE in Bio-Rad mini-protein II cell as described previously (Rao et al., 1996). After electro transfer onto PVDF membrane, the FLAG-BRCA1a fusion protein was detected with anti-FLAG M2 antibody diluted 1:100 using Western exposure chemiluminescent detection system from Clontech or ECL as described previously (Rao et al., 1996).

### Metabolic labeling of cells

Briefly, confluent 100 mm plates of HL 60 cells were labeled with  $^{32}$ P-orthophosphoric for 4 h. The cells were lysed in radioimmunoprecipitation assay buffer. Following sedimentation the supernatants were subjected to immunoprecipitation using rabbit anti BRCA1 peptide or recombinant protein antibody or preimmune serum as described previously (Rao et al., 1996). The samples were subjected to 10% SDS polyacrylamide gel electrophoresis and autoradiography. In some cases the cold HL60 cell lysates were subjected to immunoprecipitation using carboxyterminal BRCA1 peptide antibody and then subjected to Western blot analysis using phosphotyrosine antibody (Santa Cruz Biotechnology).

### Expression and purification of GST fusion protein

Expression and purification of GST fusion proteins were described previously (Rao and Reddy, 1993) except with slight modification (Frangioni and Neel, 1993). Briefly, log phase cultures of *E. coli* BL21 (DE3) LysS transformed with the pGEX 2TK-BRCA1 (aa 1–76), pGEX2T-BRCA1 (aa 1–76), pGEX 2T-E2F-1, pGEX2T-CycA, pGEX2T-cycB1, pGEX2T-cycD1, pGEX2T-E2F-4 plasmids were incubated with IPTG for 3 h. The cells were pelleted in STE buffer (Frangioni and Neel, 1993) containing 100 µg/ml lysozyme, 5 mM DTT, 1 mM PMSF and 2% Sarkosyl, sonicated on ice and centrifuged at 10 000 g for 10 min. To the supernatant Triton X-100 was added and applied to a glutathione sepharose 4B column (Pharmacia) and the GST-BRCA1 or GST-cyclins or GST-E2F fusion proteins were either left immobilized or eluted with elution buffer containing glutathione (Rao and Reddy, 1993). The GST-BRCA1 fusion proteins were labeled with  $^{32}$ P as described (Kaelin et al., 1992). In brief, the GST-TK-fusion protein probes were labeled in a 100 µl final volume containing 20 mM Tris (pH 7.5), 100 mM NaCl, 12 mM MgCl<sub>2</sub>, 10 µCi of ( $\gamma$ - $^{32}$ P) ATP, 1 µg GST-fusion protein and 100 units of cAMP dependent protein kinase (Sigma) on ice for 30 min.

### GST pull down assay

CAL-51, ZR 75-1 or HL 60 cells were labeled with  $^{35}\text{S}$ -methionine as described previously (Rao *et al.*, 1996). The cells were washed in cold phosphate buffered saline (PBS) and scrapped into 1 ml of TNN buffer (Takashima *et al.*, 1994) and lysed by rotating for 30 min at 4°C. The lysates were centrifuged at 14 000 *g* for 30 min and subjected to protein binding assay as described (Takashima *et al.*, 1994). For protein binding assay cell extracts were precleared overnight with GSH-beads and then incubated with either GST protein-conjugated GSH-beads or GST-BRCA1 (containing residue 1 to 76 of the BRCA1a protein). Proteins were incubated with GSH beads for 2 h at 4°C. The beads were then washed in TNN buffer and boiled in SDS sample buffer and loaded on a 10% SDS-PAGE. The gels were fixed, treated with enhance, dried and exposed to X-ray films. For *in vitro* binding experiments 10–20  $\mu\text{l}$  of full length *in vitro* translated BRCA1a, BRCA1b, BRCA1 (amino acids 1–182), cdc2, cdk2, cdk4, cdk5 and E2F-1 were tested for binding to GST-E2F-1 or GST-E2F-4 as described previously for GST pull down assay.

### Immunoprecipitation and in vitro kinase assays

CAL-51 cells were lysed in 1 ml TNN buffer and immunoprecipitated with recombinant BRCA1 polyclonal antibody (amino acid 1–76) as described previously (Rao *et al.*, 1996). The immunoprecipitates were washed in kinase buffer and measured for kinase activity toward histone H1 as described previously (Makela *et al.*, 1994).

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### Far Western blot analysis

Far Western blot analysis was done as described previously (Kaelin *et al.*, 1992; Singh *et al.*, 1989) except with slight modifications. In brief, after transfer the nitrocellulose membrane was washed in 1×HBB buffer (Singh *et al.*, 1989) and treated sequentially with 1×HBB buffer containing different concentrations of guanidine HCl ranging in concentration from 6 M to 0.19 M. The membrane was hybridized in Hyb 75 buffer (Kaelin *et al.*, 1992) containing 0.1 mM  $\text{ZnCl}_2$  and  $^{32}\text{P}$ -labeled GST-TK-BRCA1 protein ( $10^6$  c.p.m./ml) overnight at 4°C. Subsequently the membrane was washed in Hyb 75 buffer, air dried and exposed to X-ray film.

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# Differential transcriptional activation by the N-terminal region of BRCA1 splice variants BRCA1a and BRCA1b

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**Abstract.** The breast and ovarian cancer susceptibility gene BRCA1, is a nuclear phosphoprotein which functions as a tumor suppressor in human breast cancer cells. BRCA1 protein contains an amino-terminal zinc finger motif and a carboxy-terminal acidic region. Recently, the carboxy-terminal region of BRCA1 and the amino-terminal region of BRCA2 proteins were shown to function as transactivation domains when fused to GAL4 DNA binding domain. We have recently isolated and characterized two new naturally occurring variants of BRCA1 (BRCA1a/p110 and BRCA1b/p100) which are phosphoproteins containing phosphotyrosine that associate with E2F transcriptional factors, cyclins and cyclin dependent kinases indicating a role for BRCA1 proteins in cell-cycle regulation. Here we show for the first time that the amino-terminal region of BRCA1a (BNT) but not BRCA1b can also function as a transcriptional activator when fused to GAL4 DNA binding domain. Thus, BRCA1/1a proteins contain two autonomous transcriptional activation domains, one at the amino-terminal region (BNT) and the other at the carboxy-terminal region (BCT). BRCA1b retains only the BCT domain since it has lost part of the potential BNT domain as a result of alternative splicing. Our results also suggest the presence of an inhibitory domain at the carboxy terminal region of BRCA1 and BRCA1a proteins (BID). Thus, BRCA1b protein may function as a dominant negative variant that could regulate the transcriptional activity of BRCA1/BRCA1a proteins and hence may serve as a marker for identifying individuals with greater potential for developing breast cancer. It may be possible that loss of transcriptional activation or protein-protein interactions in patients with mutations in the amino terminal zinc finger domain could deprive the cell of an important mechanism for regulating cell proliferation leading to the development of breast cancer.

## Introduction

Breast cancer is one of the most common malignancies affecting women in the United States. The breast and ovarian cancer susceptibility gene BRCA1 was mapped by genetic linkage analysis to human chromosome 17q21.3 (1-5) establishing the presence of a breast cancer susceptibility gene. Mutations in the BRCA1 gene account for 45% of the inherited breast and ovarian cancers and are found in 90% of families with high incidence of both breast and ovarian cancers. The BRCA1 cDNA codes for a 1863 amino acid protein with an amino-terminal zinc finger domain and a carboxy-terminal acidic region typical of several transcriptional factors (3). The BRCA2 gene which also encodes a large protein was found to be mutated in 45% of familial breast cancers (6). Previously, the carboxy-terminal region of BRCA1 (7,8, Rao *et al*, unpublished data) and the amino-terminal region of BRCA2 proteins (9) were shown to activate transcription when fused to the GAL4 DNA binding domain suggesting a function for BRCA1 in transcriptional activation. Several groups have characterized the developmental patterns of expression of murine BRCA1 (10-13). BRCA1 expression was found to be high in rapidly proliferating tissues (10,11) particularly those undergoing differentiation suggesting a role for BRCA1 in cellular growth and differentiation. BRCA1 was also shown to be essential for early embryonic proliferation and development (14-16). The BRCA1 protein is a nuclear phosphoprotein (17-21) which localizes to discrete nuclear dots during S phase (19,22,23). These structures contain two additional proteins Rad51 and BARD1, both of which associate with BRCA1 *in vivo* (23,24) suggesting a role for BRCA1 in DNA repair and the maintenance of genome stability. Recently, DNA damaging agents were shown to alter the subcellular localization and phosphorylation state of p220 BRCA1 protein suggesting a role in S phase DNA damage-dependent cell cycle checkpoint response (25). Our results suggest that BRCA1 proteins accumulate in the nucleus in the absence of serum and in the cytoplasm in the presence of serum in some cells revealing a new type of regulation that may modulate the activity of the BRCA1 gene (20). Several other functions have been associated with BRCA1 for instance BRCA1 when overexpressed in breast and ovarian cancer cells results in growth inhibition both *in vitro* and *in vivo* in breast cancer cells (26, Rao *et al*, unpublished data). Conversely, inhibition of BRCA1 expression by antisense RNA in mouse fibroblasts

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**Key words:** BRCA1a; BRCA1b; transcriptional activation, zinc finger, breast cancer

or by antisense oligonucleotides in breast cancer cells resulted in transformation of mouse fibroblasts as well as increasing the rate of growth of breast cancer cells (18,27) implicating a direct role for BRCA1 in growth and tumor suppression. We have recently found the BRCA1 splice variant BRCA1a to induce apoptosis in human breast cancer cells (28) suggesting a role for BRCA1 proteins in the regulation of apoptosis. All these results seem to indicate BRCA1 to be a truly multifunctional protein.

Previously, we have isolated and characterized two new BRCA1 splice variants, BRCA1a (p110) and BRCA1b (p100) which are phosphoproteins containing phosphotyrosine (20). BRCA1a and BRCA1b proteins are localized both in the cytoplasm and nucleus and associate via their amino-terminal zinc ring finger domain with E2F transcriptional factors, cyclins/cdk complexes suggesting a function for this domain in mediating protein-protein interaction and a role for BRCA1 in cell cycle regulation (20). Recently, two other groups (29,30) have identified two BRCA1 splice variants BRCA1 $\Delta$ 672-4092 and BRCA1 $\Delta$ 11b. The molecular weight of p110 kDa BRCA1a protein is comparable to the 110 kDa BRCA1 $\Delta$ 11b (30). Since only the carboxy-terminal region of BRCA1 protein was previously tested and shown to function as a transcriptional activation domain (7,8), we investigated whether full length and various deletion mutants of splice variants BRCA1a and BRCA1b are able to activate transcription (20). Here we report for the first time that the amino-terminal region of BRCA1a but not BRCA1b can function as a transactivation domain when fused to a heterologous GAL4 DNA binding domain. Thus, BRCA1a contains two autonomous transcriptional activation domains, one at the amino-terminal region called BNT and the other at the carboxy-terminal region called BCT. BRCA1b has lost part of the amino-terminal transactivation domain as a result of alternative splicing. Our results also suggest the presence of a negative regulatory domain at the carboxy-terminal regions of BRCA1 and BRCA1a proteins. It may be possible that mutations in the zinc finger domain found in patients with breast and ovarian cancer impair this activity, suggesting that loss of transcriptional activation by BRCA1 may lead to the development of breast and ovarian cancers. Alternately BRCA1b may function as a dominant-negative regulator of the transcriptional activation function of BRCA1/BRCA1a proteins.

## Materials and methods

**Mammalian expression plasmids.** Full length and various deletions of the BRCA1a and BRCA1b coding sequences were fused in-frame with the GAL4 DNA binding domain (residues 1-147) in the GAL4 DNA binding domain vector (31) (a gift of P. Chambon). The CAT reporter plasmid 17MX2-tk-CAT contains two GAL4 binding sites linked to the CAT gene was also obtained from P. Chambon. The pCH110 plasmid containing the  $\beta$ -galactosidase gene was used as an internal control to normalize the transfection efficiency.

**Tissue culture and transient transfection.** NIH3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were plated at  $5 \times 10^5$  per 100 mm dish and transfected by the calcium phosphate co-

precipitation method (Promega) as described by the manufacturer with a total of 20  $\mu$ g DNA (3  $\mu$ g of GAL-4 DNA binding domain vector plasmid or GAL-4 BRCA1a/1b plasmids, 4  $\mu$ g of 17MX2-tk-CAT reporter plasmid, 5  $\mu$ g of pCH110 plasmid and 8  $\mu$ g of Gem3 carrier plasmid). Cells were harvested at 48 h-63 h post-transfection. They were then lysed and the extracts assayed for  $\beta$ -galactosidase and CAT activities as described (18). The CAT assays were quantitated using a Fuji PhosphorImager. The experiments were repeated at least six times.

## Results and Discussion

Full length cDNAs and several amino and carboxy-terminal deletions of BRCA1a and BRCA1b were subcloned in-frame with the GAL4 DNA binding domain vector obtained from P. Chambon (31). The GAL4 fusion constructs (Fig. 1) were co-transfected into NIH3T3 fibroblast cells, along with the reporter plasmid 17MX2-tk-CAT that contains two GAL4 binding sites linked to the chloramphenicol acetyltransferase gene (CAT) and an internal control plasmid CH110. Both full-length GAL4-BRCA1a and GAL4-BRCA1b plasmids failed to show CAT activity (Fig. 2) when compared to the GAL4 vector control. Two carboxy-terminal truncated BRCA1a plasmids, one lacking exons 21-24 and the other lacking exons 16-24, failed to show transactivation. Further carboxy-terminal deletion of BRCA1a (deletion of aa 263-1863) but not BRCA1b plasmid (deletions of aa 263-1863) showed ~7-10 fold CAT activity. These results suggest the presence of a transactivation domain at the amino-terminal region of BRCA1a (BNT) and an inhibitory domain (BID) at the carboxy-terminal region of the BRCA1a protein (Fig. 1). However, further carboxy-terminal deletion of exons 9 and 10 of BRCA1a and exons 9 and 10 alone when fused to the GAL4 DNA binding domain failed to show significant CAT activity. The fact that exons 9 and 10 as such failed to show activity indicates that additional sequences N-terminal and C-terminal to this region are also required for transcriptional activation. All these results suggest that apart from the carboxy-terminal region of BRCA1, the amino-terminal region of BRCA1a/BRCA1 (aa 1-262), which also includes the BRCA1 zinc finger domain, can also function as a transcription activation domain (BNT). The scenario we observe with BRCA1a/1b proteins parallel what is observed in Elk-1 and  $\Delta$ Elk-1 proteins where SRF binds to the ETA-1 domain of the Elk-1 protein thereby unmasking the ETA-2 domain facilitating phosphorylation and activation of transcription (32-35), in tumor suppressor gene p53 where MDM2 inhibits p53 transactivation by concealing its activation domain (36) and in yeast where GAL80 may inhibit GAL4 function by a similar phenomenon (37,38). Based on our results (this paper) and results of others (7,8, Rao *et al.*, unpublished data), we have proposed a working hypothetical model as to how BNT and BCT transcriptional activation domains of BRCA1 might function *in vivo* (Fig. 3). In short, the BNT domain of BRCA1 protein can function *in vivo* independent of BCT, since truncation of the BCT domain results in significant transcriptional activation. The BCT domain may function either in the absence of BNT (as seen in BRCA1b protein) or in the presence of protein factors. It may be possible that such

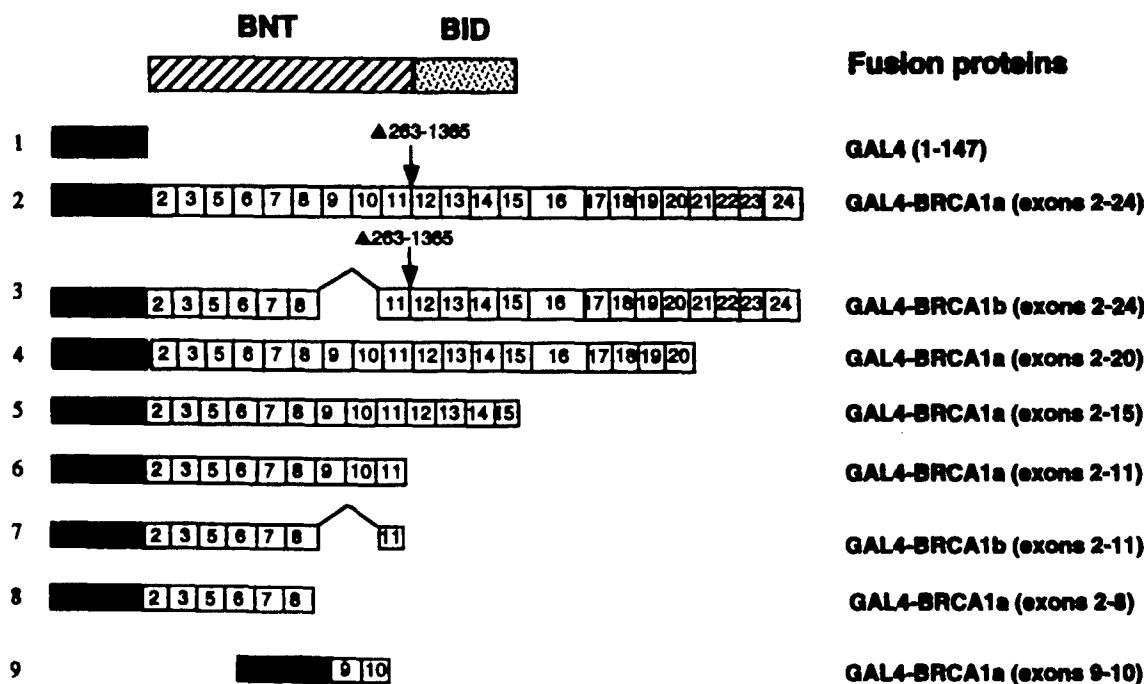


Figure 1. Transcriptional activation by the N-terminus of BRCA1 and BRCA1a proteins. Full length and various portions of the BRCA1a and BRCA1b coding regions were fused in-frame with the GAL4 DNA binding domain (residues 1-147). BNT, BRCA1 N-terminal activation domain; BID, BRCA1 inhibitory domain. The numbers above the arrows show the amino-acids that are deleted in BRCA1a/b and their derivatives. The amino-acids are numbered according to gene bank BRCA1 sequence (3) (#U14680).

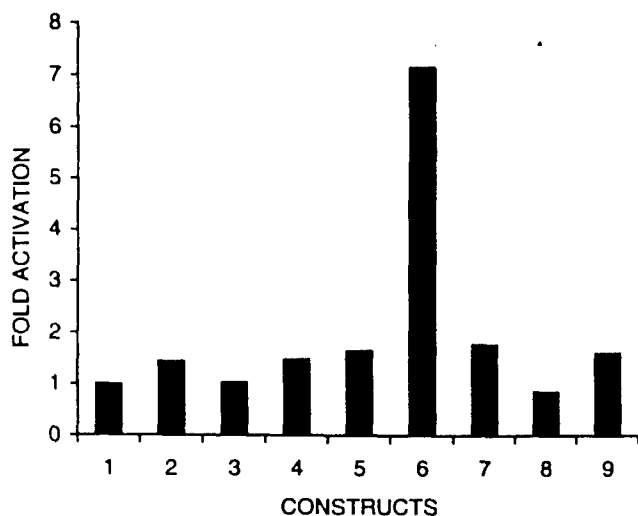


Figure 2. The constructs were co-transfected with the reporter construct 17MX2-lk-CAT into NIH3T3 cells and CAT activity was measured at 48 h-63 h following transfection. The numbers represent the plasmids shown in Fig. 1. The results are an average of at least six independent experiments. The CAT activity shown represents fold activation compared with control GAL4 DNA-binding domain vector alone.

factor(s) might bind to the BNT domain of BRCA1a protein, somehow conceal its activation domain and in the process unmask the BCT domain facilitating activation of transcription. The BNT domain may be functioning as a negative regulatory transcription activation domain for BCT. Although earlier results have indicated that the carboxy-

terminal segment of BRCA1 associates with the RNA pol II holoenzyme (39), it may be possible that the amino-terminal transactivation domain (BNT) could modulate this interaction. Furthermore, our recent results indicate that both BRCA1a and BRCA1b proteins interact *in vitro* and *in vivo* with CBP co-activator (Cui *et al*, unpublished data). The binding of BRCA1a to CBP is weak when compared to BRCA1b suggesting that the BNT domain of BRCA1a may be functioning as an inhibitory domain for binding to CBP protein. Future experiments will be designed to address these issues. Previously, the zinc finger domain of BRCA1 was shown to interact both *in vitro* and *in vivo* with BARD1 (24) and *in vitro* with E2F transcriptional factors and cyclins/cdk complexes (20), which suggested a function for this domain in mediating protein-protein interactions and a role for BRCA1 in cell cycle regulation. The BRCA1 zinc finger domain is the location of some of the most frequently occurring mutations linked to breast and ovarian cancers. One BRCA1 mutation contains a frame shift in exon 2 (188 del 111) that removes the zinc finger domain from the protein. The 185 del AG mutation, which is the most common BRCA1 mutation [about 1% of Ashkenazi Jewish individuals (40)], disrupts the BRCA1 gene product at the first residue of the C<sub>3</sub>HC<sub>4</sub> domain. Some frequent missense mutations Cys61, Gly, Cys 64, Gly and Cys 64 Tyr also disrupt the zinc finger domain structure. It may be possible that loss of transcriptional activation or protein-protein interactions in patients with mutations in the zinc finger domain could deprive the cell of an important mechanism for regulating cell proliferation leading to the development of breast cancer.

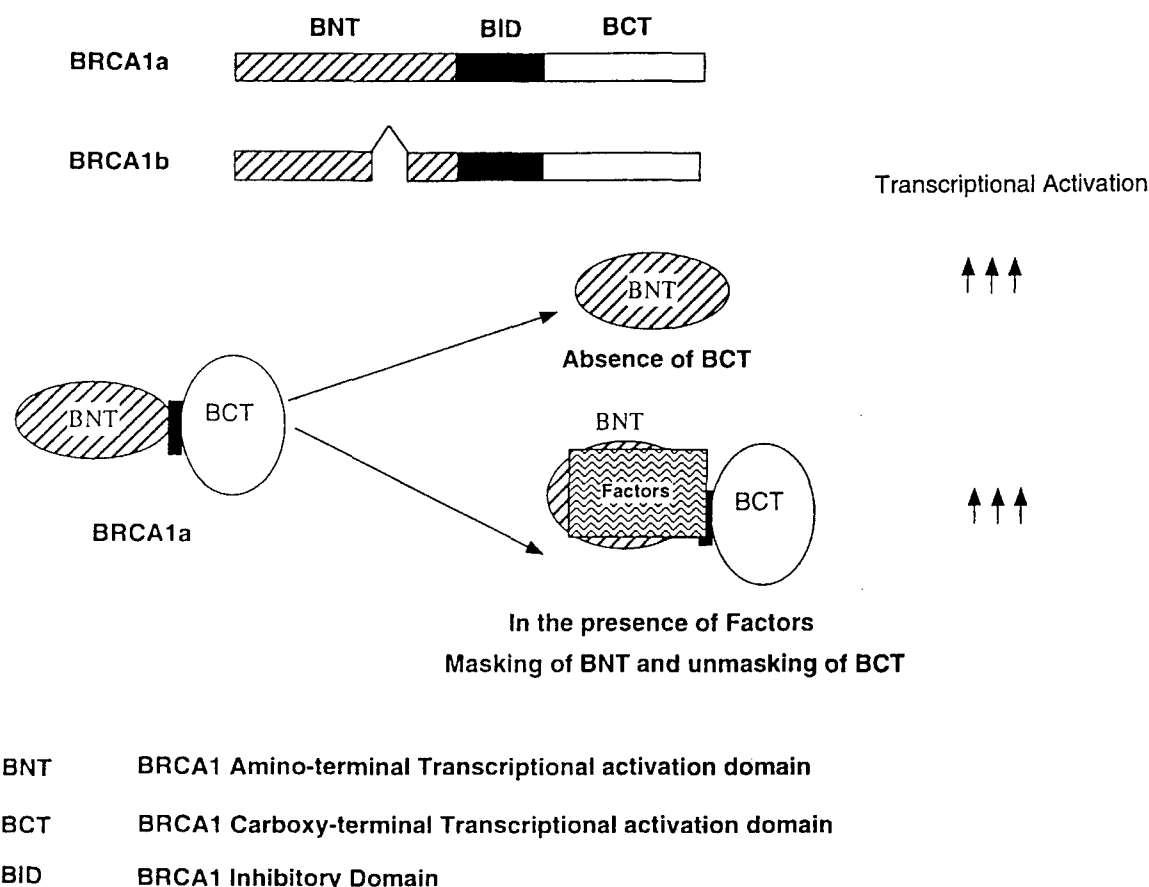


Figure 3. Hypothetical model as to how the two transcriptional activation domains BNT and BCT of the BRCA1 proteins might function *in vivo*.

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# BRCA1 splice variants BRCA1a and BRCA1b associate with CBP co-activator

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**Abstract.** The tumor suppressor gene BRCA1, is a nuclear phosphoprotein which associates with RNA polymerase II holoenzyme. CBP is a component of the holoenzyme. Previously, we have characterized two new BRCA1 splice variants BRCA1a/p110 and BRCA1b/p100. In the present study, the carboxy-terminal domain of transcription factor CBP interacts both *in vivo* and *in vitro* with full length BRCA1a and BRCA1b proteins as demonstrated by mammalian two-hybrid assays, co-immunoprecipitation/Western blot studies, GST binding assays and histone acetyl transferase (HAT) assays of BRCA1 immunoprecipitates from human breast cancer cells. Our results suggest that one of the mechanisms by which BRCA1 proteins function is through recruitment of CBP associated HAT/FAT (transcription factor acetyl-transferase) activity for acetylation of either themselves or general transcription factors or both to specific promoters resulting in transcriptional activation.

## Introduction

BRCA1, a familial breast and ovarian cancer susceptibility gene is mutated in 45% of the inherited breast and ovarian cancers and 90% of families with high incidence of both breast and ovarian cancers (1-3). The BRCA1 cDNA codes for a 1863 amino acid protein with an amino-terminal zinc finger domain and a carboxy-terminal acidic region typical of several transcriptional factors. Recently, the carboxy-terminal region of BRCA1 was shown to activate transcription (4,5, Rao, unpublished data) when fused to a GAL4 DNA binding domain. Our results suggest the presence of an autonomous

transcriptional activation domain at the amino-terminal region of BRCA1 proteins (Cui JQ and Rao VN, unpublished data) similar to BRCA2 (6). Earlier results indicated that intact BRCA1 associates with the RNA polymerase II holoenzyme and the carboxy-terminal region of BRCA1 is important for holoenzyme binding (7). The BRCA1 protein is a nuclear phosphoprotein (8-12) which localizes to nuclear dots during S phase (10,13,14) and associates with Rad51 and BARD1 suggesting a role for BRCA1 in DNA repair and the maintenance of genome stability (14,15). Recently DNA damaging agents were shown to alter the subcellular localization and phosphorylation of p220 BRCA1 protein suggesting a role in S phase DNA damage dependent cell cycle check point response (16). Our results suggest that BRCA1 proteins accumulate in the nucleus in the absence of serum and in the cytoplasm in the presence of serum in some cells revealing a new type of regulation that may modulate the activity of BRCA1 gene (11). Several other functions such as growth and tumor suppression (9,17,18), induction of apoptosis (19) have been associated with BRCA1 proteins indicating BRCA1 to be truly a multifunctional protein. Previously, we have isolated and characterized two new BRCA1 splice variants, BRCA1a (p110) and BRCA1b (p100) (11). BRCA1b protein has lost part of the potential amino-terminal transcriptional activation domain as a result of alternative splicing (Cui JQ and Rao VN, unpublished data). BRCA1a and BRCA1b proteins are phosphoproteins containing phosphotyrosine that are localized both in the cytoplasm and nucleus and associate with E2F transcriptional factors, cyclins/cdk complexes suggesting a role for BRCA1 proteins in cell-cycle regulation (11).

## Materials and methods

**GST pull down assay.** GST-CBP1 (residues 461-662) and GST-CBP2 (residues 1621-1877) were expressed and purified from *Escherichia coli* as reported previously (11). For *in vitro* binding assay <sup>35</sup>S-methionine labeled *in vitro* translated full length BRCA1a and BRCA1b (Rao, unpublished data) proteins were diluted in TNN buffer (20) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF), 10 µl/ml leupeptin, 3% aprotinin and 1 mM sodium orthovanadate. The solutions were precleared with GSH-beads for 1 h and then incubated with GST-CBP1 beads or GST-CBP2 beads for 2 h at 4°C. The beads were then washed five times in TNN buffer and heated

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**Abbreviations:** CBP, CREB-binding protein; HAT, histone acetyl transferase; GST, glutathione S-transferase; FAT, transcription factor acetyl-transferase

**Key words:** BRCA1, CBP co-activator, histone acetyl transferase, transcription factor acetyl-transferase

in SDS sample buffer and loaded on a 10% SDS PAGE. The gels were fixed, treated with enhance, dried and exposed to X-ray films or exposed and scanned using a Fuji BioImaging analyzer.

**IP-HAT assays.** CAL51 cells were cultured in 75 cm (2) culture flasks in DMEM medium containing 10% FBS, 0.6 µg/ml bovine insulin,  $5 \times 10^{-3}$  µg/ml transferrin, 1% penicillin-streptomycin. IP-HAT assay was done essentially as described (21) except with slight modifications. Briefly, cell pellets were collected by trypsinization and washed once in PBS. The cells were lysed in 1 ml of lysis buffer IPH (21) except that IGEPAL CA-630 (Sigma) was substituted for NP-40. The lysis mixture was then incubated on ice for 20 min and cleared by centrifugation at 13,000 rpm for 10 min at 4°C. Either CBP A-22 (Santa Cruz Biotechnology) or BRCA1 D-20 (Santa Cruz Biotechnology) or BRCA1a recombinant polyclonal antibody or Myc polyclonal antibody (Santa Cruz Biotechnology) were added to 1 ml of extract and incubated at 4°C for 2 h. A 1:1 mixture (15 µl total) of protein A-sepharose/protein G-sepharose was added and the mixture rotated overnight at 4°C. The immunocomplexes were centrifuged and washed three times with 1 ml lysis buffer IPH. 1.25 µl of 20 mg/ml histone or BSA, 1 µl of  $^3\text{H}$ -acetyl Co A (4.3 Ci/mMol Amersham) were added to the immunocomplexes to a total volume of 30 µl with lysis buffer IPH. HAT assays were performed at 30°C for 45 min to 1 h. The reactions were spotted onto Whatman P-81 filters, washed five times with 0.2 M  $\text{Na}_2\text{CO}_3$  15 min each, dried and counted in a Beckman liquid scintillation counter.

**Immunoprecipitation/Western blot assay.** CAL51 cells ( $1 \times 10^6$ ) were collected by washing in PBS and treating with trypsin. For preparing total cell extracts the cells were lysed in 1.0 ml RIPA buffer and the lysate was centrifuged at 14,000 rpm for 30 min at 4°C. To the supernatant normal rabbit IgG and protein A-sepharose CL-4B (Pharmacia) was added and rotated gently at 4°C overnight. The supernatant was then incubated with BRCA1 polyclonal antibody (Santa Cruz SC-641) or CBP polyclonal antibody (Santa Cruz SC-369) for an hour at 4°C. The proteins were then crosslinked to protein A-sepharose CL-4B for 2 h at 4°C. The beads were then washed twice with RIPA buffer and 1/10 detergent buffer [150 mM NaCl, 40 mM Tris-HCl (pH 7.5), 0.1% NP40, 0.01% sodium deoxycholate, 0.01% SDS, 1 mM EDTA, 1 mM EGTA]. The beads were boiled with 40 µl of SDS-PAGE loading buffer for 5 min and electrophoresed either on a 6% SDS-PAGE (for CBP analysis) or 10% SDS-PAGE (for BRCA1 analysis) and subjected to Western blot analysis as described previously (9). Following incubation of the membranes with CBP or BRCA1 antibodies, the proteins were detected using a Western exposure chemiluminescent detection system from ECL as suggested by the manufacturer (Amersham).

**Plasmid construction and transfections.** Full length BRCA1a and BRCA1b cDNAs (Rao, unpublished data) were subcloned in frame with the GAL4 DNA binding domain vector obtained from P. Chambon (22). PHK Gal4-FOS, pHK3n CBP1 VP16 (expressing CBP 461-662 in-frame with the VP16AD), pHK3n CBP2 VP16 (expressing CBP 1621-1877 in-frame with the VP16 (AD) G5E1bCAT and pHK3nVP16 plasmids were a

gift from T. Kouzarides. PSGVP16 (expressing the VP16 activation domain) plasmid has been described previously (33). U2OS human osteosarcoma cells were maintained in McCoy's 5A supplemented with 10% FCS and grown at 37°C (5%  $\text{CO}_2$ ). These cells were transfected using the calcium phosphate co-precipitation method (Promega) as described previously (9). At 48 h post transfection cell extracts were prepared from transfected cells and subjected to  $\beta$ -galactosidase and CAT assays. The CAT assays were quantitated with a Fuji PhosphoImager. The experiments were repeated at least 5-6 times. For studying the ectopic expression of GAL4 BRCA1a and GAL4 BRCA1b fusion proteins, COS-7 cells were transfected with 20 µg of GAL4 DNA binding domain vector DNA or 20 g of GAL4 BRCA1a DNA or GAL4 BRCA1b DNA using the calcium phosphate co-precipitation method as described above. At 48-56 h post transfection cell extracts were prepared and ~90 µg of protein was loaded on an 8% SDS PAGE and subjected to Western blot analysis using anti GAL4 (DBD) polyclonal antibody (Sant Cruz Biotechnology, Inc.).

## Results and Discussion

Recently, BRCA1 protein was shown to associate and co-purify with RNA polymerase II holoenzyme (7). Since CREB binding protein (CBP) is a component of the holoenzyme (23), we speculated whether BRCA1 could interact directly with co-activator CBP which would bring the holoenzyme into play. In the present study, both full length BRCA1a and BRCA1b proteins interact with the carboxy-terminal domain of CBP protein (1620-1877) as demonstrated by mammalian two-hybrid assays. Direct physical interaction of CBP with BRCA1a/BRCA1b proteins were shown by glutathione S-transferase fusion protein binding assays, co-immunoprecipitation/Western blot studies and HAT activity assays of BRCA1 immunoprecipitates from breast cancer cells. All these results suggest that BRCA1 proteins interact with CBP and targeting CBP-associated HAT/FAT activity to specific promoters may be one of the mechanisms by which BRCA1 proteins function.

CBP is a well characterized co-activator which is a component of the RNA polymerase II holoenzyme (23). CBP interacts with the phosphorylated form of transcription factor CREB and activates transcription (24). A closely related co-factor p300 was identified on the basis of its interaction with adenovirus E1A (25). Both CBP and p300 interact with a variety of cellular transcriptional activators that include nuclear hormone receptors, CREB, c-Jun/v-Jun, c-Myb/v-Myb, stat-1, stat-2, c-fos, Myo D, NFkBp65, Elk-1, p53 and basal components of the transcriptional apparatus (24,26-28).

To demonstrate interaction between CBP and BRCA1a/BRCA1b proteins *in vitro* and *in vivo*, we have used three different approaches: GST fusion protein pull down assays, a mammalian two-hybrid system and immunoprecipitation/Western blotting method. The CBP protein has at least two domains which bind to transcriptional factors: a region containing residues 461-662 (designated CBP1) which is required for binding CREB, c-Jun, cMyb; a region containing residues 1621-1877 (designated CBP2) which is required for interactions with E1A, c-Fos and TF11B (24,26). GST fusion proteins containing CBP1 and CBP2 domains were expressed as described (11). To examine whether BRCA1a/1b proteins

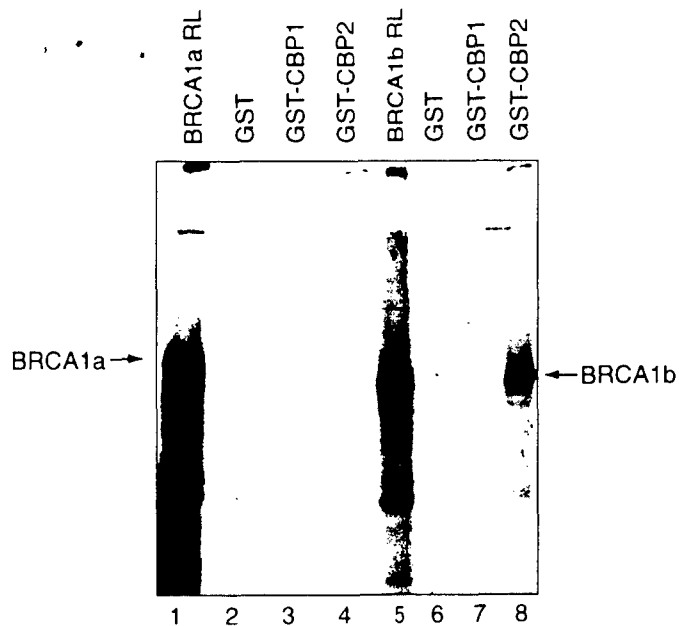


Figure 1. Direct physical interactions of CBP with BRCA1a and BRCA1b *in vitro*. GST, GST-CBP1 or GST-CBP2 were incubated with *in vitro* translated  $^{35}\text{S}$ -labeled BRCA1a or BRCA1b and subjected to GST pull down assay. 1  $\mu\text{l}$  of the *in vitro* translated BRCA1a and BRCA1b proteins were run as controls. Arrows represent the *in vitro* translated BRCA1a (p110) and BRCA1b (p100) proteins.

directly interact with CBP, *in vitro* binding was performed with *in vitro* translated BRCA1a/1b and GST-CBP proteins. *In vitro* transcription and translation of BRCA1a and BRCA1b in the presence of  $^{35}\text{S}$ -methionine generated radiolabeled BRCA1a and BRCA1b polypeptides of 110 and 100 kDa respectively (Fig. 1, lanes 1 and 5). These radio-labeled proteins were passed through GST-CBP1, GST-CBP2 and a GST control column. Both BRCA1a and BRCA1b were found to bind to GST-CBP2. As no BRCA1a and BRCA1b were detected in the eluates from the GST, GST-CBP1 columns (Fig. 1, lanes 2, 3, 6 and 7), these results suggest that both BRCA1a/BRCA1b proteins bind specifically to GST-CBP2. The BRCA1a splice variant binds weakly to GST-CBP2 compared to BRCA1b similar to what was observed earlier with binding to E2F and cyclins (11). It may be possible that the amino-terminal transcriptional activation domain present in BRCA1a may be serving as an inhibitory domain for binding to CBP protein. Taken together these results suggest that BRCA1a/1b interact physically with CBP, since GST-CBP2 binds to *in vitro* translated BRCA1a/1b proteins.

Having established that the CBP2 domain of CBP can directly associate with intact BRCA1a and BRCA1b proteins *in vitro*, we next studied the interaction *in vivo* using the mammalian two-hybrid system. We subcloned the full length BRCA1a and BRCA1b cDNA's in frame with the GAL4 DNA binding domain vector (22). The CBP1 VP16 and CBP2 VP16 plasmids in which the CBP1 and CBP2 domains have been fused to the activation domains of VP16 were obtained from T. Kouzarides (26). U20S human osteosarcoma cells or COS cells were co-transfected with CBP1 VP16 or CBP2 VP16 and GAL4 BRCA1a or GAL4 BRCA1b and G5E1bCAT as reporter. Fig. 2A shows that in the two hybrid *in vivo*

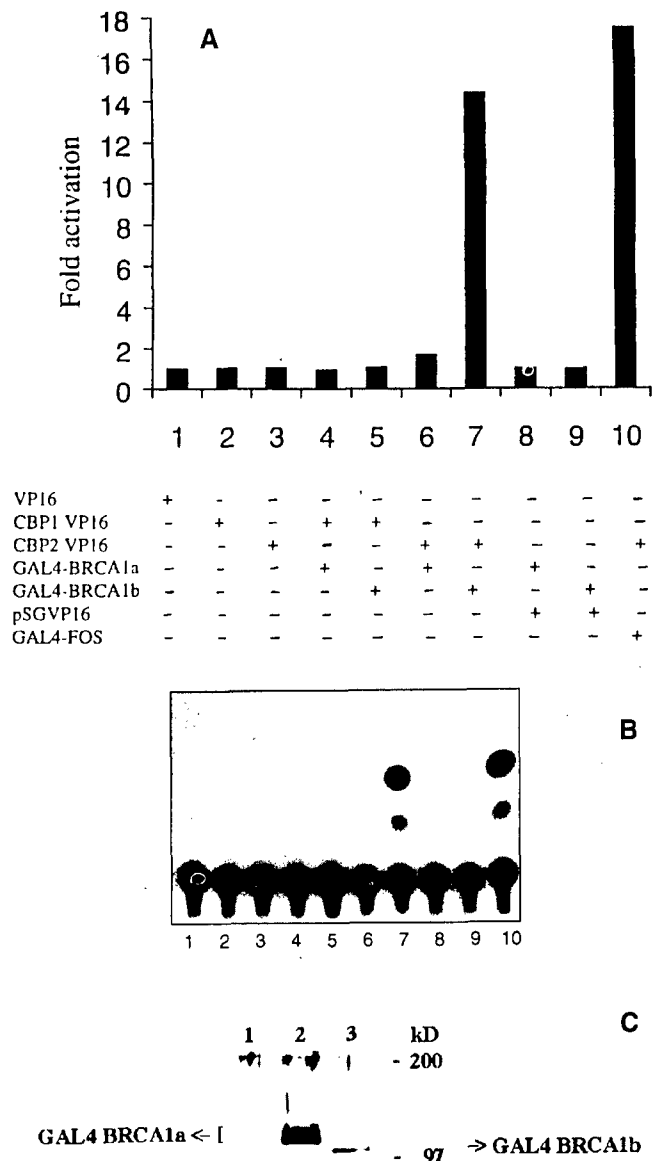


Figure 2. Full length BRCA1a and BRCA1b interact with the carboxy-terminal domain of CBP (CBP2) in a mammalian two-hybrid system. A, U20S human osteosarcoma cells were co-transfected with 4  $\mu\text{g}$  pG5E1BCAT reporter plasmid, 1  $\mu\text{l}$  of VP16 expression plasmids (pHK3nVP16 or pSGVP16 or pHK3nCBP1VP16 or pHK3nCBP2VP16) and 1  $\mu\text{l}$  of GAL-4 expression plasmids (GAL-4 BRCA1a or GAL-4 BRCA1b or GAL-4 Fos). Total DNA was kept constant at 20  $\mu\text{g}$ . The CAT activity shown represents fold activation compared with the VP16 vector alone. The activity of the vector is normalized to a value of 1. B, The numbers represent the columns shown in A. Data that was used to calculate the fold activity represented in A. The experiments were repeated at least six times. C, Ectopic expression of BRCA1a and BRCA1b in COS cells. Extracts from control cells (lane 1), GAL4 BRCA1a-transfected cells (lane 2) or GAL4 BRCA1b-transfected cells (lane 3) were subjected to 8% SDS-PAGE and Western blot analysis with anti-GAL4 (DBD) polyclonal antibody. The doublet bands seen in lane 2 represent phosphorylated forms of GAL4 BRCA1a fusion proteins and the band in lane 3 represents GAL4 BRCA1b fusion protein.

interaction experiments, the CBP2VP16 protein simulates GAL4 BRCA1b activity specifically (15 fold). This activation is not seen with the CBP1VP16 hybrid protein nor with the pSGVP16 vector alone (Fig. 2A and B). The stimulation observed with GAL4 BRCA1b was comparable to that seen with GAL4-Fos and CBP2 VP16 (~17 fold). In the same experiment, GAL4 BRCA1a showed marginal stimulation in



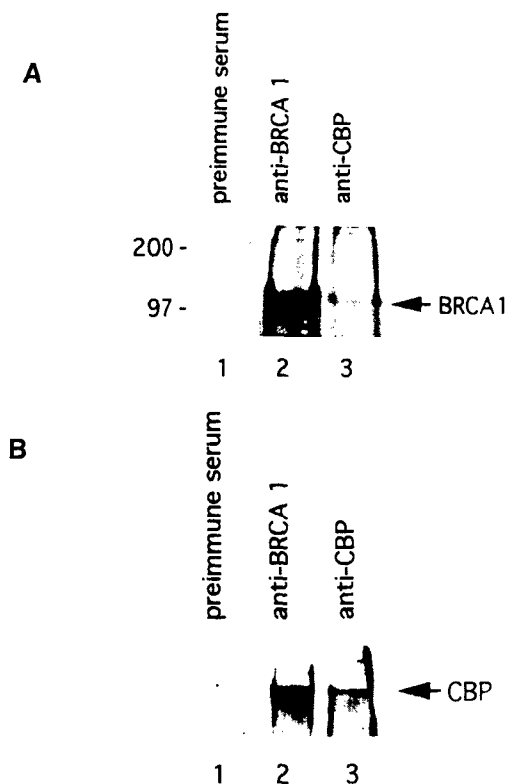


Figure 3. *In vivo* interaction of CBP with BRCA1 proteins in CAL51 cells. A, Western blot analysis of CAL51 whole cell extract with anti-BRCA1 after immunoprecipitation with preimmune serum (lane 1), anti-BRCA1 (lane 2) and anti-CBP (lane 3). B, Immunoblot with anti-CBP after immunoprecipitation of CAL51 whole cell extract with preimmune serum (lane 1), anti-BRCA1 (lane 2) and anti-CBP (lane 3). The BRCA1 arrow in A (lane 3) represents BRCA1b (p100).

presence of CBP2 VP16 (Fig. 2A and B), consistent with the *in vitro* binding results (Fig. 1). We studied the expression of GAL4 BRCA1a fusion protein in transiently transfected COS cells by Western blot analysis using a GAL4 DNA binding domain specific polyclonal antibody. The GAL4 BRCA1a protein was expressed at higher levels compared to GAL4 BRCA1b fusion protein (Fig. 2C) thus ruling out the possibility that the weak interaction observed *in vivo* with VP16 CBP2 is due to inappropriate expression of BRCA1a protein. It may be possible that the amino-terminal transcriptional activation domain present in BRCA1a may be serving as an inhibitory domain for binding to CBP protein.

Interactions between CBP and BRCA1 proteins *in vivo* were further demonstrated by using immunoprecipitation/immunoblot analysis. To study these associations in a physiologically relevant environment, cell extracts from human breast cancer cells CAL-51 were immunoprecipitated with anti-CBP or anti-BRCA1 antibodies and then Western blotted with anti-BRCA1 or anti-CBP antisera. The anti-BRCA1 antiserum has been used previously to detect BRCA1a/1b splice variants by immunoprecipitation and Western blot analysis (9,11,19). The CAL-51 cell lysates immunoprecipitated with anti-CBP antisera showed an immunoreactive band at 100 kDa that was recognized with anti-BRCA1 antisera (Fig. 3A, lanes 2 and 3). In a reciprocal experiment, immunoprecipitation of CAL-51 cell lysates with anti-BRCA1 antibody showed an immunoreactive band that was recognized by antibodies against CBP (Fig. 3B, lanes 2 and 3). These results suggest that an

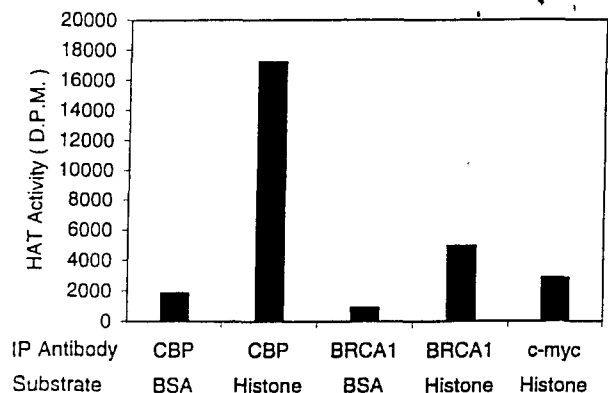


Figure 4. BRCA1 antibody immunoprecipitates HAT activity in an IP-HAT assay. CAL51 whole-cell extracts were immunoprecipitated with CBP or BRCA1 or Myc antibodies as indicated. The Myc antibody was used as a negative control (21). The immune complexes were washed extensively and assayed for their ability to acetylate BSA or histones.

association between BRCA1b and CBP occurs in intact cells. The precise mechanism by which transcription factors stimulate the transcriptional machinery through contacting CBP/p300 is unclear. Recently, p300/CBP and P/CAF were shown to possess histone acetyl transferase activities (21,29,30) suggesting that recruiting CBP/p300 associated HAT activity to specific promoters may be one of the mechanisms by which transcription factors function.

We next tested the BRCA1 immunoprecipitates from CAL-51 cells for HAT activity. Our results demonstrate that these immunocomplexes show HAT activity that is specific for histones (Fig. 4). Antibodies against nuclear proteins like Myc only precipitated a background non-specific activity (Fig. 4). The HAT activity associated with the BRCA1 immunoprecipitates can either be due to the acetylase activity complexed specifically with CBP (21) or there could be a possibility that BRCA1 proteins could themselves possess intrinsic HAT activity similar to Gcn5, CBP/p300 and TAF250 (21,26,29-32). In summary, our results demonstrate the binding of BRCA1 proteins to CBP and suggest that BRCA1 may carry HAT activity when associated with CBP similar to E1A protein (21). The question whether the function(s) (transactivation, protein-protein interactions, etc.) of BRCA1 proteins can be modified by acetylation similar to that observed for tumor suppressor gene p53 and whether BRCA1 proteins possess intrinsic HAT activity awaits further experimentation (30). The binding of BRCA1a to CBP is weak or almost negligible when compared to BRCA1b indicating that the amino terminal transactivation domains present in BRCA1a protein (Rao, unpublished data) may inhibit the binding to CBP. Furthermore, based on these results, we can speculate that the levels of BRCA1a/1b proteins may be critical for identifying individuals with greater risk for developing breast cancer. Taken together these results suggest that one of the mechanisms by which BRCA1 proteins function is through recruitment of acetyl transferase containing CBP co-activators for acetylation of either themselves or general transcription factors or both to specific promoters resulting in transcriptional activation. Alternatively, BRCA1 proteins may also serve as adaptor proteins which could bridge the association between CBP co-activator and RNA polymerase II complexes. It may

be possible that lack or impaired binding of disrupted BRCA1 proteins or variant BRCA1 proteins to CBP in patients with mutations could deprive the cell of an important mechanism for regulating cell proliferation leading to the development of breast and ovarian cancers.

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## SHORT REPORT

## The BRCA2 is a histone acetyltransferase

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Patients carrying mutations in *BRCA1* or *BRCA2* tumor suppressor genes have shown to have high risk in developing breast and ovarian cancers. Two potential functions of *BRCA2* were proposed which includes role in the regulation of transcription and also in DNA repair. Forty-five-amino acid region encoded by exon 3 of *BRCA2* was shown to have transcriptional activation function. Recent studies of the several enzymes involved in acetylation and deacetylation of histone residues have revealed a possible relationship between gene transcriptional activation and histone acetylation. Since *BRCA2* appear to function as a transcriptional factor, we have tested for Histone acetyl transferase (HAT) activity of *BRCA2*. Here, we present evidence that *BRCA2* has intrinsic HAT activity, which maps to the amino-terminal region of *BRCA2*. Our results demonstrate that *BRCA2* proteins acetylate primarily H3 and H4 of free histones. These observations suggest that HAT activity of *BRCA2* may play an important role in the regulation of transcription and tumor suppressor function.

**Keywords:** *BRCA2*; histone acetyl transferase; protein-protein interaction; tumor suppressor

Alterations in *BRCA1* and *BRCA2* tumor suppressor genes have been shown to be involved in 90% of familial breast cancers (Newman *et al.*, 1988; Miki *et al.*, 1994; Easton *et al.*, 1993; Wooster *et al.*, 1994; Wooster and Stratton, 1995). Recent studies revealed that both *BRCA1* and *BRCA2* are involved in ovarian and prostate cancers. Interestingly, *BRCA2* was found to be more associated with male breast cancer compared to *BRCA1* (Wooster *et al.*, 1994). Patients with *BRCA2* mutations were also found to be at a higher risk with a variety of other cancers including carcinomas of pancreas, prostate and colon (Thorlacius *et al.*, 1996; Phelan *et al.*, 1996; Gudmundsson *et al.*, 1995; Tonin *et al.*, 1995). The *BRCA2* gene is composed of 27 exons and encodes a protein of 3418 amino-acids with no significant homology to any known protein (Wooster *et al.*, 1995; Bork *et al.*, 1996). *BRCA2* and *BRCA1* proteins have been shown to interact with Rad 51 which suggests that they play a role in DNA repair (Scully *et al.*, 1997; Sharan *et al.*, 1997; Zhang *et al.*, 1998). *BRCA1* was also shown to induce apoptosis suggesting that *BRCA* proteins may

play a role in the regulation of apoptosis of cells (Shao *et al.*, 1996; Rao *et al.*, 1996). It remains to be seen whether *BRCA2* plays a similar role in apoptosis.

Interestingly, both *BRCA1* and *BRCA2* gene products are regulated in a cell cycle-dependent manner and have a potential transactivation function (Rajan *et al.*, 1996; Vaughn *et al.*, 1996; Chapman and Verma, 1996; Montero *et al.*, 1996; Milner *et al.*, 1997; Wang *et al.*, 1997; Cui *et al.*, 1998a). Recently, we have shown that *BRCA1* proteins interact with transcriptional co-activator CBP suggesting that *BRCA1* has a role in the regulation of transcription (Cui *et al.*, 1998b). Exon 3 of *BRCA2* was found to have weak homology with transcriptional factor *c-jun* and also shown to activate transcription in mammalian cells (Milner *et al.*, 1997). These results suggest that *BRCA2r* has a role in the regulation of gene expression.

Recent studies of the several enzymes involved in acetylation and deacetylation of histone residues have revealed a possible relationship between gene transcriptional activation and histone acetylation (Brownell *et al.*, 1996; Parthun *et al.*, 1996; Yang *et al.*, 1996; Orgyko *et al.*, 1996; Mizzen *et al.*, 1996; Roth and Allis, 1996; Wade and Wolffe, 1997; Pazin and Kadonaga, 1997; Wolffe, 1997). This view is supported by the identification of Histone acetyl transferase (HAT) activity associated with several transcription factors including p300/CBP, GCN5-related factors, p/CAF, SRC-1 and TAF<sub>II</sub> 250. These results suggest that some transcriptional activators operate by disrupting the nucleosomal structure through acetylation of histones leading to the activation of gene expression.

Here, we report for the first time that the amino-terminal region of *BRCA2* has intrinsic HAT activity from which it may be inferred that *BRCA2* joins the above list of transcriptional activators/factors that possess HAT activity. This intrinsic *BRCA2*-HAT activity may play a key role in the tumor suppressor function of *BRCA2*.

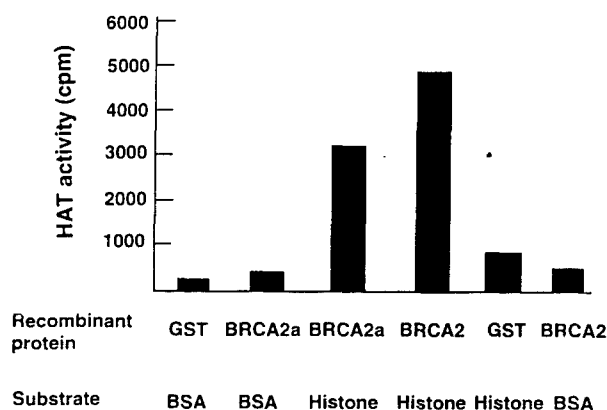
Recently, we have cloned an alternatively spliced isoform, *BRCA2a*. This variant *BRCA2a* lacks a transcriptional activation domain (exon 3) as a result of alternative splicing (our unpublished results). In order to test the HAT activity of *BRCA2*, we have expressed the amino-terminal region of *BRCA2* (aa 1–500) and its isoform *BRCA2a* (aa (1–18)-(105–500)) as GST-fusion proteins in bacteria by cloning appropriate *BRCA2* cDNA fragments into a GST expression vector (Our unpublished results). Purified recombinant proteins of *BRCA2* and *BRCA2a* were assayed for histone acetyl transferase activity. Amino-terminal domains of both *BRCA2* and *BRCA2a* clearly demonstrated histone acetyl transferase activity (Figure 1). Control samples where *BRCA2* or *BRCA2a* was replaced with bovine serum albumin (BSA) showed no

significant HAT activity. Similar control experiments where histones were replaced by BSA (lysine rich nonhistone protein) also failed to show significant acetyl transferase activity. This suggests that BRCA2 proteins show specific acetyl transferase activity to histones (Figure 1). Therefore, we conclude that BRCA2 *per se* is a histone acetyl transferase. Since the amino-terminal region of BRCA2 and BRCA2a show HAT activity, we conclude that the exon 3 (aa 18–105) responsible for the transactivation function of BRCA2 is not needed for HAT activity function. These results suggest that the transactivation and HAT functional domains of BRCA2 do not overlap with each other (Figure 2).

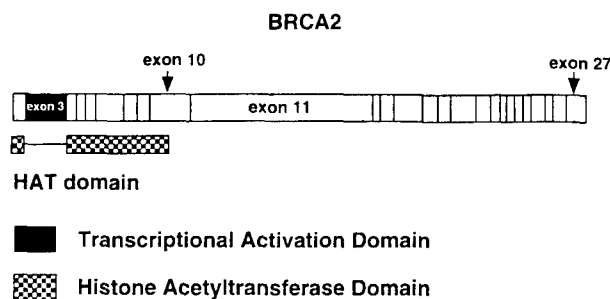
In order to determine which histones are acetylated by BRCA2 proteins, we have carried out HAT assay with free core histones and analysed the resulting products by SDS-polyacrylamide gel electrophoresis followed by fluorography. Our results demonstrate that BRCA2 proteins acetylated primarily H3 and H4 of free histones (Figure 3). We have also confirmed these results using individual free histones (data not shown).

In order to determine the HAT activity associated with BRCA2 *in vivo*, we have carried out immunoprecipitation HAT assay. Immunoprecipitation of BRCA2 from whole cell extracts was tested for acetyl

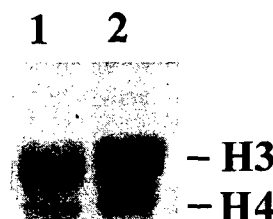
transferase activity. Our results demonstrate that immunoprecipitated BRCA2 carries acetylase activity specific for histones (Figure 4). These *in vitro* and *in vivo* results support the conclusion that BRCA2 has intrinsic HAT activity. It is conceivable that as in the case of CBP/p300 (which shows intrinsic HAT activity), transcriptional activators recruit BRCA2 and utilize its intrinsic HAT activity for their transcriptional activation properties. It is also possible that BRCA2 also in turn recruits other factors (like p/CAF, p300/CBP) that possess distinct HAT activity and thereby disrupt the nucleosomal structure through their cooperative HAT activity. This results in the activation of gene expression interacts with CBP both *in vitro* and *in vivo*. Therefore, it is tempting to speculate that the target genes of BRCA2 play key roles in growth inhibition, differentiation and apoptosis. Identification of these genes may provide clues to the role of BRCA2 in neoplasia. Because of its large size, it is conceivable that BRCA2 has multi-cellular functions which include DNA repair, transcriptional activation, HAT etc. It is possible that BRCA2/Rad 51 Complex may use HAT activity to disrupt the nucleosomal structure to recognize



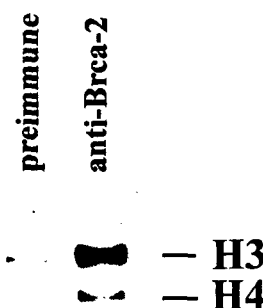
**Figure 1** BRCA2 has intrinsic HAT activity. The amino-terminal region of BRCA2 (aa 1–500) and BRCA2a ((1–18)–(105–500)) were expressed as GST fusion proteins in bacteria and subsequently purified. HAT assays were carried out as described but with slight modification (Bannister and Kouzarides, 1996; Herrera *et al.*, 1997). Approximately 50–100 ng of GST-fusion proteins of BRCA2 and BRCA2a were used to acetylate 15 µg of core histones (Boehringer Mannheim) in the presence of [<sup>3</sup>H]acetyl CoA



**Figure 2** Schematic representation of the functional domains of BRCA2



**Figure 3** Acetylation profile and Substrate specificity of BRCA2 and BRCA2a. Recombinant BRCA2a (aa 1–500) (lane 1) and BRCA 2 ((1–18)–(105–500)) (lane 2) were incubated with core histones as described above. [<sup>14</sup>C]acetylated histones were separated on SDS–PAGE and detected by autoradiography



**Figure 4** BRCA2 antibodies immunoprecipitate HAT activity. Immunoprecipitations (IP) were performed from NIH3T3 whole cell extract with either anti-BRCA2 (Santa Cruz) antibody or pre-immune serum. These IPs were tested for their ability to acetylate free histones as described above. Pre-immune serum served as a negative control

damaged DNA for DNA repair. Patients with mutations in HAT and/or transactivation domains of BRCA2 may show a loss of gene expression which are critical for growth inhibition and differentiation and result in a subset of familial breast and prostate cancers. One can use BRCA2-HAT assay for screening patients with BRCA2 mutations.

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## SHORT REPORT

Induction of apoptosis by Elk-1 and  $\Delta$ Elk-1 proteins

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**Elk-1, an ets related gene codes for at least two splice variants Elk-1, which regulates *c-fos* transcription and  $\Delta$ Elk-1, both of which function as transcriptional activators. To investigate the role of Elk-1 and  $\Delta$ Elk-1 proteins in apoptosis; we have developed rat fibroblast cell lines and human breast cancer cell lines expressing Elk-1 and  $\Delta$ Elk-1. The expression of Elk-1 and  $\Delta$ Elk-1 proteins in the Elk-1/ $\Delta$ Elk-1 transfectants were analysed by immunofluorescence, immunohistochemistry, and Western blot analysis. The Elk-1 unlike  $\Delta$ Elk-1 transfectants showed a shortened and flattened morphology compared to the parental cells. We have found that calcium ionophore treatment of Rat-1 Elk-1, MCF-7 Elk-1, Rat-1  $\Delta$ Elk-1 and MCF-7  $\Delta$ Elk-1 transfectants resulted in programmed cell death. These results indicate that constitutive expression of Elk-1 and  $\Delta$ Elk-1 proteins triggers apoptosis in Rat-1 fibroblasts and breast cancer cells when treated with calcium ionophore.**

**Keywords:** Elk-1;  $\Delta$ Elk-1; apoptosis; calcium ionophore; breast cancer; MCF-7; Rat-1

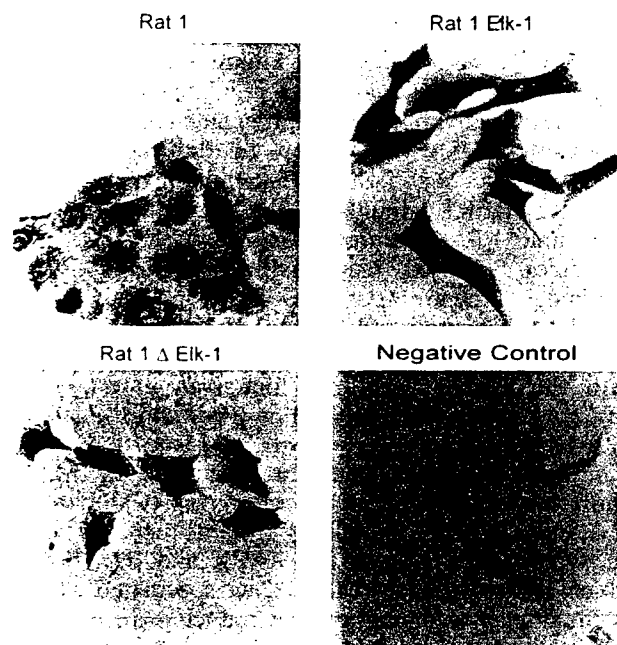
The Elk-1 gene belongs to the ets family of ternary complex factors (TCFs), i.e. Elk-1, SAP1, and NET ERP/SAP2/Elk-3 (Rao *et al.*, 1989; Hipkind *et al.*, 1991; Giovane *et al.*, 1994; Lopez *et al.*, 1994; Dalton and Treisman, 1992; Price *et al.*, 1995; Nozaki *et al.*, 1996). The Elk-1 gene codes for at least two alternately spliced products Elk-1 (Rao *et al.*, 1989) and  $\Delta$ Elk-1 (Rao and Reddy, 1993) which function as transcriptional activators (Rao and Reddy, 1992; Bhattacharya *et al.*, 1993), are substrates for MAP kinases (Rao and Reddy, 1993; Marias *et al.*, 1993; Hill *et al.*, 1993) and JNK protein kinases (Gupta *et al.*, 1996; Whitmarsh *et al.*, 1995). As mentioned earlier, the Elk-1 protein is a TCF which in association with serum response factor (SRF) forms a ternary complex on the serum response element (SRE) of the *c-fos* promoter and regulates *c-fos* transcription (Hipkind *et al.*, 1991). The TCF's which includes Elk-1 have three domains with similar sequences and functions. The ets domain mediates DNA binding, the SRF interaction domain interacts with SRF to form a ternary complex with the *c-fos* SRE and the C-terminal domain activates transcription

upon phosphorylation by MAP kinases (Rao *et al.*, 1989; Rao and Reddy, 1992; Dalton and Treisman, 1992; Janknecht *et al.*, 1993, 1994; Marias *et al.*, 1993; Giovane *et al.*, 1994; Hipkind *et al.*, 1994; Kortenjann *et al.*, 1994; Lopez *et al.*, 1994; Hill *et al.*, 1995; Price *et al.*, 1995; Whitmarsh *et al.*, 1995) and JNK kinases (Gupta *et al.*, 1996). Thus Elk-1 represents a key link between signal transduction and induction of gene transcription.

The Gag-Myb-Ets fusion protein, identified in the avian acute leukemia virus E26 was shown to inhibit apoptosis and induce erythroid differentiation in hematopoietic cells (Athanasίου *et al.*, 1996). Similarly the Ets-1 proto-oncogene was shown to be required for the normal survival and activation of B and T cells while an Ets-1 splice variant was shown to induce apoptosis in human colon cancer cells indicating a role in apoptosis (Bories *et al.*, 1995; Muthusamy *et al.*, 1995; Huang *et al.*, 1997). Recently, *erg* and *fl-1* proteins were shown to inhibit apoptosis (Yi *et al.*, 1997). Mitogen activated extracellular response kinase kinase kinase (MEKK), a serine-threonine kinase that regulates phosphorylation and activation of mitogen-activated protein kinases (MAPK) was shown to induce cell death (Johnson *et al.*, 1996). Inducible expression of activated MEKK stimulated the transactivation of c-Myc and Elk-1 (Johnson *et al.*, 1996). To date, molecules involved in signaling apoptosis include ceramide (Jimenez *et al.*, 1995), Rho, Ras, c-Myc, p53, E1A (Canman and Kastan, 1995), c-Jun (Evan *et al.*, 1992), Fas (Wang *et al.*, 1994), proteins associated with the TNF $\alpha$  receptor (Chinnaiyan *et al.*, 1995), BRCA1 (Shao *et al.*, 1996), Fos (Preston *et al.*, 1996), E2F-1 (Shan *et al.*, 1996), c-Myc and c-Jun transcription factors which are regulated by MAPK phosphorylation also induce apoptosis (Canman and Kastan, 1995). Since Elk-1 protein regulates c-Fos oncogene and is a target for MAPK and JNK both of which are activated by MEKK, we speculated whether it could play a similar role in inducing an apoptotic response. In this study, we have developed rat fibroblast cell lines and human breast cancer cell lines expressing Elk-1 and  $\Delta$ Elk-1 proteins. Our results suggest that constitutive expression of Elk-1 and  $\Delta$ Elk-1 induce apoptosis in both Rat-1 and MCF-7 cells.

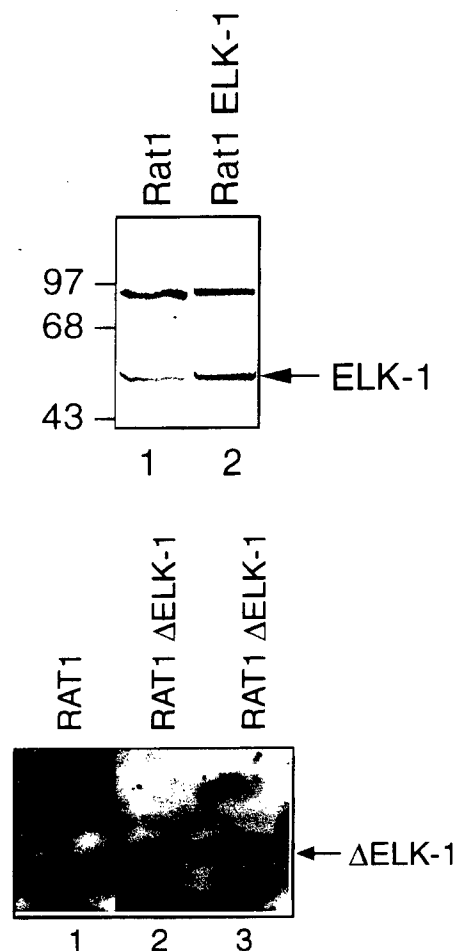
In an attempt to study the function of Elk-1 and  $\Delta$ Elk-1 proteins in the regulation of apoptosis, we have transfected Rat-1 fibroblasts and MCF-7 breast cancer cells with pcDNA expression vector pcDNA expression vectors containing human Elk-1 and

$\Delta$ Elk-1 cDNA and obtained stable G418 resistant cell lines expressing Elk-1 and  $\Delta$ Elk-1 proteins. The morphology of the Elk-1 transfectants were different from that of the parental Rat-1 fibroblast and MCF-7 cells. The Elk-1 transfectants were slow growing and appeared to be shorter and flatter compared to the parental Rat-1 MCF-7 cells. The morphology of the  $\Delta$ Elk-1 transfectants were similar to that of the parental cells. We analysed the expression of Elk-1 and  $\Delta$ Elk-1 proteins in Elk-1 and  $\Delta$ Elk-1 transfectants by indirect immunofluorescence analysis, immunoperoxidase staining (Figure 1) and Western blot analysis (Figure 2) using Elk-1 polyclonal antibody as described previously (Shao *et al.*, 1996; Wang *et al.*, 1997). The nuclear and cytoplasmic staining was brighter and stronger in both Elk-1 and  $\Delta$ Elk-1 transfectant cells compared to parental Rat-1 fibroblast cells suggesting higher levels of expression of Elk-1 and  $\Delta$ Elk-1 proteins in transfectant cells (Figure 1). Western blot analysis revealed a significant increase in the levels of expression of Elk-1 and  $\Delta$ Elk-1 proteins when compared to the parental Rat-1 cells (Figure 2). Similarly, we have also observed significant increase in the expression of Elk-1 and  $\Delta$ Elk-1 proteins in MCF-7 Elk-1 and MCF-7  $\Delta$ Elk-1 transfectants by immunoperoxidase staining and Western blot analysis (data not given). Calcium ionophore A23187 is known to induce apoptosis in thymocytes and neurons and previously, we have shown A23187 to induce apoptosis in BRCA1a



**Figure 1** Detection of Elk-1 and  $\Delta$ Elk-1 proteins in Rat-1, Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 cells by immunohistochemistry. Full length Elk-1 (Rao *et al.*, 1989) and  $\Delta$ Elk-1 (Rao and Reddy, 1993) cDNAs were subcloned into pcDNA expression vectors. Purified DNA (20  $\mu$ g) of pcDNA expression vector or vector containing Elk-1  $\Delta$ Elk-1 cDNAs were transfected into Rat-1 and MCF-7 cells by calcium phosphate precipitation method using the Invitrogen kit as described previously (Rao *et al.*, 1996). Rat-1, Elk-1 and  $\Delta$ Elk-1 transfectant cells were cultured in chamber slides and processed for immunohistochemistry using an Elk-1 carboxyterminal peptide antibody as described previously (Rao *et al.*, 1996; Shao *et al.*, 1996)

transfected NIH3T3 and MCF-7 cells (Cohen *et al.*, 1984; Joseph *et al.*, 1993; Shao *et al.*, 1996). This led us to examine apoptosis in Rat-1 Elk-1, MCF-7 Elk-1, Rat-1  $\Delta$ Elk-1 and MCF-7  $\Delta$ Elk-1 transfectants after A23187 treatment. Rat-1, Rat-1 Elk-1, Rat-1  $\Delta$ Elk-1 and MCF-7, MCF-7 Elk-1 and MCF-7  $\Delta$ Elk-1 cells were treated with calcium ionophore A23187 for 24–48 h and the cell cycle distribution was determined by flow cytometry with propidium iodide staining method. Histogram of the DNA content and the percentage of cells in G1, S and G2 plus M phase of the cell cycle were evaluated using EPICS profile analyser. The Elk-1 and  $\Delta$ Elk-1 transfectant cells showed accelerated rates of apoptosis (Ap value 49% for Rat-1 Elk-1, 66% for MCF-7 Elk-1, 42% for Rat-1  $\Delta$ Elk-1 and 28% for MCF-7  $\Delta$ Elk-1 cells) in the presence of calcium ionophore (Figure 3a and b)



**Figure 2** Western blot analysis of Rat-1 cells stably transfected with Elk-1 and  $\Delta$ Elk-1 using an anti-C-terminal Elk-1 antibody. Rat-1, Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 cells were harvested by washing in PBS and treating with trypsin. For preparing total cell extracts the cells were lysed in RIPA buffer and subjected to Western blot analysis as described previously (Rao *et al.*, 1996). For Western blotting analysis ~50  $\mu$ g of cell extract in SDS sample buffer were loaded on a 10% SDS-PAGE in Bio-Rad mini-protein II cell as described previously (Rao *et al.*, 1996). After electrophoresis, the proteins were transferred electrophoretically to nitrocellulose membrane (Amersham). Immunodetection of Elk-1 protein was performed with a polyclonal anti-Elk-1 peptide antibody (Santa Cruz Biotechnology, I-20) diluted 1:100 using ECL Western exposure chemiluminescent detection system from Amersham

whereas the control Rat-1 fibroblasts and MCF-7 cells showed lower levels of apoptosis under identical conditions (Ap value 16% and 5% respectively).

Measurement of apoptosis through the sub G1 peak in the DNA histogram gives no distribution between viable and dead cells since all the cells are fixed. We, therefore, studied the viability of Rat-1 cells, Rat-1 Elk-1, Rat-1  $\Delta$ Elk-1, MCF-7 cells, MCF-7 Elk-1 and MCF-7  $\Delta$ Elk-1 cells cultured in the presence of calcium ionophore by MTT metabolic assay. In this assay, viable cells will cleave MTT to produce formazan using mitochondrial enzyme succinate tetrazolium reductase and the quantity of formazan dye produced correlates directly to the number of viable cells. As shown in Figure 3c, only 35% of Rat-1 Elk-1 cells and 33% of Rat-1  $\Delta$ Elk-1 cells remained viable upon exposure to calcium ionophore for 24 h, compared to nearly 80% of control Rat-1 cells. Similarly, (Figure 3d) only 21% of MCF-7 Elk-1 and 24% of MCF-7  $\Delta$ Elk-1 cells survived upon exposure to calcium ionophore for 24 h, compared to nearly 40% of control MCF-7 cells. These results suggest that both Elk-1 and  $\Delta$ Elk-1 proteins induce death in Rat-1 and MCF-7 cells.

Elk-1 and  $\Delta$ Elk-1 transfected Rat-1 cells were cultured in the presence of calcium ionophore to induce apoptosis and the incidence of cell death was determined by phase contrast microscopy after staining the cultures with Hoechst 33258. The nuclei of Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 cells showed strong chromatin condensation and nuclear degradation into small, spherical nuclear particles of condensed chromatin characteristic of apoptosis, whereas the parental Rat-1 cells did not show any significant change in the staining pattern (Figure 3e).

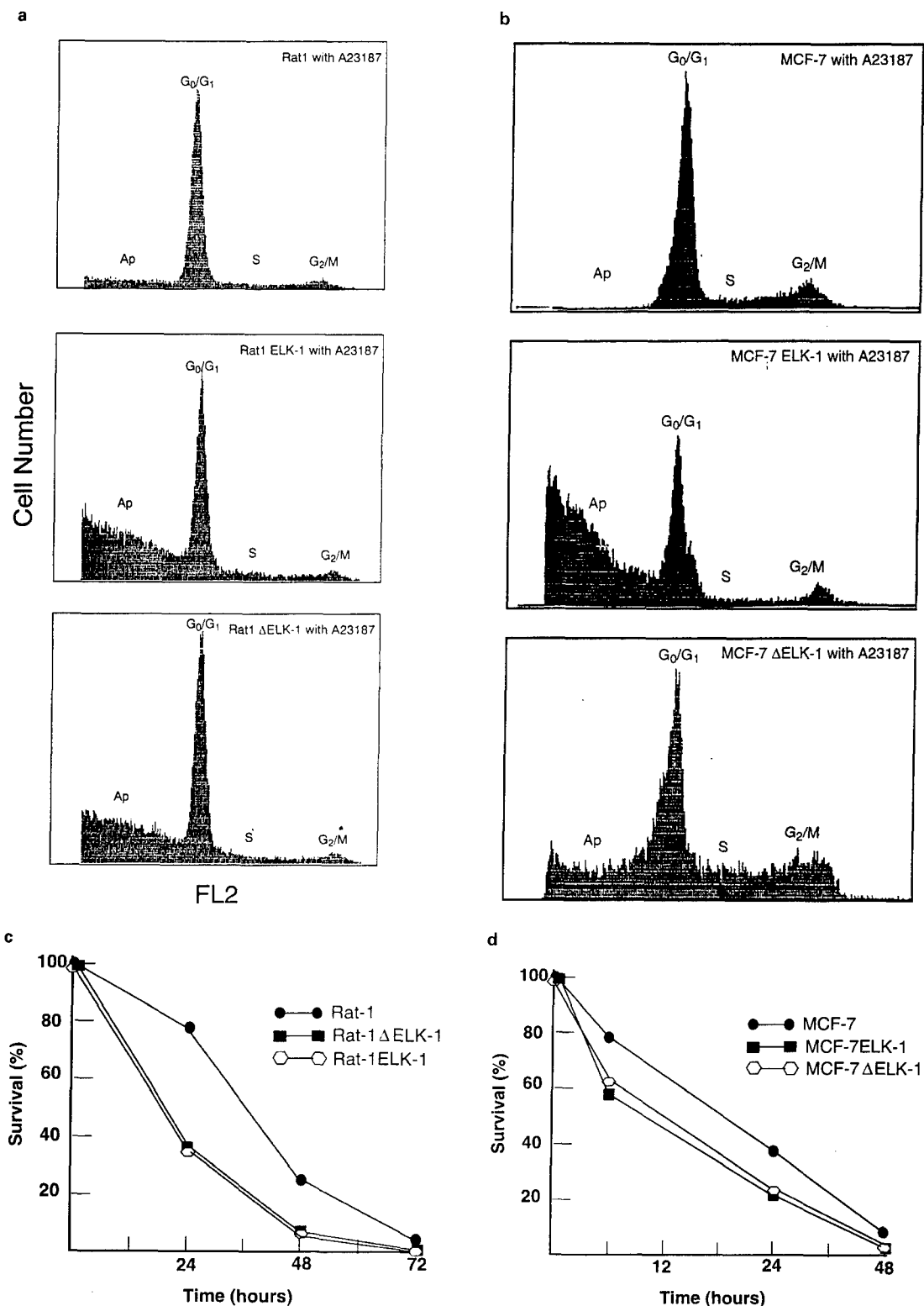
The induction of apoptosis in the Elk-1 and  $\Delta$ Elk-1 transfectants upon treatment with calcium ionophore was further confirmed by analysis of DNA fragmentation. The DNA of Elk-1 and  $\Delta$ Elk-1 cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder typical of apoptosis whereas the parental Rat-1 MCF-7 cells showed no significant DNA degradation (Figure 3f and g). These results suggested that calcium ionophore induces apoptosis more readily in Rat-1 Elk-1, MCF-7 Elk-1, Rat-1  $\Delta$ Elk-1, MCF-7  $\Delta$ Elk-1 cells than control Rat-1 MCF-7 cells and overexpression of Elk-1 and  $\Delta$ Elk-1 induces apoptosis in breast cancer cells. All the above results shown for a single clone of Rat-1 Elk-1 or Rat-1  $\Delta$ Elk-1 or MCF-7 Elk-1 or MCF-7  $\Delta$ Elk-1 cells have been reproducibly obtained with several other independent clonal isolates of Rat-1 MCF-7-Elk-1 and Rat-1 MCF-7 $\Delta$ Elk-1 cells. From our data it appears that Elk-1 is more effective in causing cell death than  $\Delta$ Elk-1 when cells are exposed to A23187. These differences in the apoptotic potential of Elk-1 and  $\Delta$ Elk-1 proteins does not appear to be due to differences in the levels of expression of these proteins but may be due to the inherent differences in their physiological functions, since Elk-1 is a ternary complex factor and *c-fos* regulator unlike  $\Delta$ Elk-1 protein.

*c-fos* transcription is induced rapidly by a wide array of extracellular stimuli, including growth factors, cytokines, neurotransmitters, ion fluxes, phorbol esters and UV irradiation. Growth-factors induce *c-fos* transcription by stimulating phosphorylation of transcriptional factor TCF/Elk-1 by MAPKs while UV irradiation and MEKK1 activation strongly activate two other MAPKs JNK1 and JNK2, which also stimulate Elk-1 transcriptional activity and phosphorylation. In short UV irradiation and MEKK1 activation stimulates Elk-1 activity through JNK activation and growth factors induce Elk-1 phosphorylation, *c-fos* transcription through ERK activation. Recently, MEKK which phosphorylates and activates MAPK, JNK and SAPK's was shown to regulate signal transduction pathways that contribute to the apoptotic response (Johnson *et al.*, 1996). Apoptosis is a highly regulated process of cell death that is characterized by cell shrinkage, chromatin condensation and cellular DNA fragmentation (Searle *et al.*, 1982). The process of programmed cell death appears to be regulated by proteins that also function in proliferation and differentiation. For example, both *c-Myc* and *c-fos* which are immediate early gene products have been shown to induce apoptosis (Canman and Kastan, 1995; Preston *et al.*, 1996). The gag-Myb-Ets fusion protein, *c-ets-1* proto-oncogene, and two ets superfamily members namely *erg* and *fl-1* have been shown to inhibit apoptosis whereas *ets-1* splice variant has been shown to induce apoptosis indicating a role for the ets family of genes in apoptosis (Athanasίου *et al.*, 1996; Bories *et al.*, 1995; Muthusamy *et al.*, 1995; Yi *et al.*, 1997; Huang *et al.*, 1997). Our findings on the Elk-1 proteins is consistent with a role for the ets related genes in cell death.

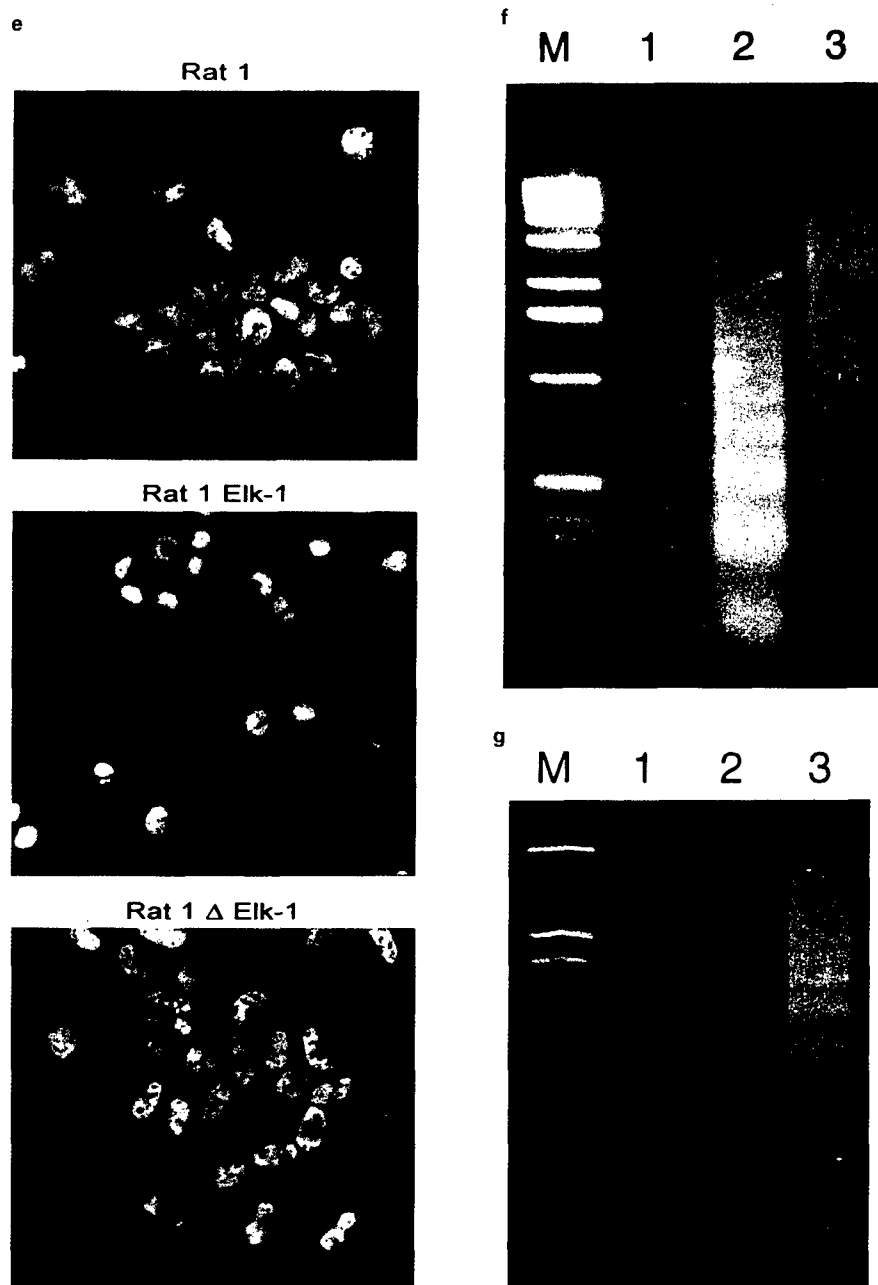
In summary our results demonstrate for the first time, a role for Elk-1 and  $\Delta$ Elk-1 proteins in mediating a cell death response characteristic of apoptosis. Thus Elk-1 and  $\Delta$ Elk-1 proteins induce apoptosis similar to Rho, Ras, *c-Myc*, *c-jun*, Fas, BRCA1, Fos, p53, E1A, E2F-1, etc (Canman and Kastan, 1995; Evan *et al.*, 1992; Wang *et al.*, 1994; Shao *et al.*, 1996; Preston *et al.*, 1996; Shan *et al.*, 1996). The precise mechanism by which Elk-1 induces cell death remains to be investigated. Since Elk-1 functions as a transcriptional regulator, it may be either activating death inducing genes like *fos* (Preston *et al.*, 1996), Bad, Bax, Bak, Bcl-xL, etc, or repressing death inhibiting genes like Bcl-2 (Oltvai and Korsmeyer, 1994; Reed, 1994), Bcl-xL, Mcl-1, A1, Bag-1, etc, leading to apoptosis.

Alternatively, Elk-1 may activate apoptosis inducing proteins or target apoptosis inhibiting proteins through direct protein-protein interactions. Elk-1 could behave similar to the *c-myc* oncogene which is implicated both in the control of normal cellular proliferation and apoptosis (Evan *et al.*, 1992). Recent evidence suggests that decrease in normal cell death is a characteristic of breast cancers (Thompson, 1995). Future efforts will be directed towards delineating the mechanisms (such as induction of Bcl-2 family members or caspases or JNK pathways) by which Elk-1 and  $\Delta$ Elk-1 proteins induce apoptosis.





**Figure 3** Elk-1 and  $\Delta$ Elk-1 proteins induce apoptosis in Rat-1 and MCF-7 cells after calcium ionophore treatment. (a) Flow cytometric analysis of Rat-1 cells induced to undergo apoptosis by calcium ionophore A23187 treatment for 48 h. (b) Flow cytometric analysis of MCF-7 cells triggered to undergo apoptosis by A23187 treatment for 24 h. Subconfluent Rat-1, Rat-1 Elk-1, MCF-7, MCF-7 Elk-1 and MCF-7  $\Delta$ Elk-1 cells were incubated in their respective media with or without 20  $\mu$ M calcium ionophore A23187. After 24–48 h, both adherent and nonadherent cells were pooled, washed in PBS and fixed in 80% cold ethanol at  $-18^{\circ}\text{C}$  overnight. Cells were stained with propidium iodide (20  $\mu\text{g}/\text{ml}$ ) and incubated with 20  $\mu\text{g}/\text{ml}$  of RNAase A at  $37^{\circ}\text{C}$  for 30 min. Samples were analysed using a EPICs profile analyser. Histograms showing the total DNA content as FL2 vs Cell numbers are shown. (c) Time course of A23187 induced cell death in Rat-1, Rat-1 Elk-1, and Rat-1  $\Delta$ Elk-1 cells. Percentage of surviving cells were measured by modified MTT assay.  $5 \times 10^4$  cells/well were seeded into 24 well culture dishes and incubated at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  for 24 h. The medium was removed and the cells were cultured in complete media containing 20  $\mu\text{M}$  calcium ionophore A23187 (Sigma). At different times, 0.1 ml of MTT (5.0 mg/ml in PBS) solution was added into each well and incubated at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  for another 3 h. The medium was discarded, 0.5 ml of 0.04 HCl-isopropanol was added into each well and kept at room temperature for 20 min. Then 0.5 ml of PBS was added and quantitative by measurement of



absorbance at 570 nm. The difference of absorbance before and after the treatment with A23187 represent the number of surviving cells. (d) Time course of A23187 induced cell death in MCF-7, MCF-7 Elk-1 and MCF-7  $\Delta$ Elk-1 cells. The percentage of surviving cells was measured by modified MTT assay. (e) Chromatin condensation shown morphologically by Hoescht staining of Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 cells induced to undergo cell death by calcium ionophore for 48 h. Rat-1, Rat-1 Elk-1 cells and Rat-1  $\Delta$ Elk-1, cells treated with or without 20  $\mu$ M calcium ionophore for 24–48 h were cultured on glass cover slips and fixed in methanol:glacial acetic acid (3:1) at  $-18^{\circ}\text{C}$  for 30 min. The cells were washed in PBS and stained with 8  $\mu$ g/ml Hoescht 33258 for 5 min in dark. The cover slips were rinsed in water and mounted with fluorescence mounting media. The cells were visualized and photographed under the fluorescence microscope. (f) DNA fragmentation induced by Elk-1 and  $\Delta$ Elk-1 overexpression in Rat-1 cells. 1, Rat-1 cells treated with calcium ionophore; 2, Rat-1 Elk-1 cells treated with calcium ionophore; 3, Rat-1  $\Delta$ Elk-1 cells treated with calcium ionophore. (g) DNA fragmentation induced by Elk-1 and  $\Delta$ Elk-1 overexpression in MCF-7 cells. Lane 1, MCF-7 cells treated with calcium ionophore; 2, MCF-7  $\Delta$ Elk-1 cells treated with calcium ionophore; 3, MCF-7 Elk-1 cells treated with calcium ionophore. This assay was modified from the one previously described (Kondo *et al.*, 1994). Briefly, subconfluent Rat-1, Rat-1 Elk-1 and Rat-1  $\Delta$ Elk-1 cells were treated with calcium ionophore A23187. After 48 h, both adherent and nonadherent cells were pooled ( $1 \times 10^6$ ) and washed once with PBS. The cell pellets were lysed in 1.0 ml of a buffer containing 10 mM Tris-HCl, 10 mM EDTA and 0.2% Triton X-100 (pH 7.5). After 10 min on ice, the lysates were centrifuged (13 000 g) for 10 min at  $4^{\circ}\text{C}$  in an Eppendorf microfuge. The supernatant (containing RNA and fragmented DNA, but not intact chromatin) was extracted first with phenol:chloroform:isoamylalcohol, then with chloroform:isoamylalcohol (24:1). The aqueous phase was made to 300 mM sodium chloride and the nucleic acids were precipitated with two volumes of ethanol. The pellet was rinsed with 70% ethanol, air dried and dissolved in 40  $\mu$ l of TE buffer (pH 8.0). Following digestion of RNA with RNAase A (100  $\mu$ l/ml, at  $37^{\circ}\text{C}$  for 30 min), the samples were electrophoresed on 2% agarose gel. DNA was then visualized after ethidium bromide staining. DNA fragmentation analysis of MCF-7, MCF-7 Elk-1 and MCF-7  $\Delta$ Elk-1 were done as described above except for the treatment of A23187 (20  $\mu$ M) for 24 h.

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Genetics. Allegheny-Singer Research Institute, Pittsburgh, and at the Kimmel Cancer Institute, Philadelphia. This work was supported in part by NIH grants CA57322, CA50507 and DAMD17-94-J-4280 by US Army Medical Research grant to VNR and NIH grant CA58642 to ESPR.

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## SHORT REPORT

The second BRCT domain of BRCA1 proteins interacts with p53 and stimulates transcription from the p21<sup>WAF1/CIP1</sup> promoter

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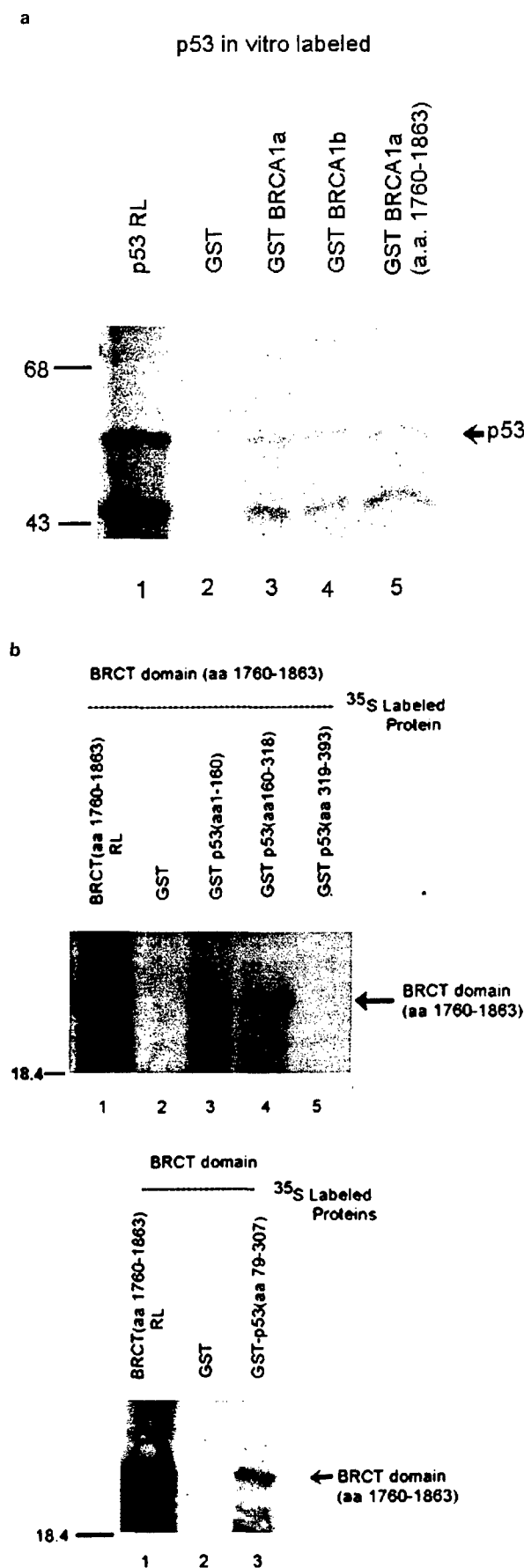
Inherited mutations in the breast and ovarian cancer susceptibility gene BRCA1 are associated with high risk for developing breast and ovarian cancers. Several studies link BRCA1 to transcriptional regulation, DNA repair, apoptosis and growth/tumor suppression. BRCA1 associates with p53 and stimulates transcription in both p53 dependent and p53-independent manners. BRCA1 splice variants BRCA1a (p110) and BRCA1b (p100) associates with CBP/p300 co-activators. Here we show that BRCA1a and BRCA1b proteins stimulate p53-dependent transcription from the p21<sup>WAF1/CIP1</sup> promoter. In addition, the C-terminal second BRCA1 (BRCT) domain is sufficient for p53 mediated transactivation of the p21 promoter. Previous studies emphasized the importance of the BRCT domain, which shows homology with p53 binding protein (53BP1), in transcriptional activation, growth inhibition and tumor suppression. Our findings demonstrate an additional function for this domain in protein–protein interaction and co-activation of p53. We also found that BRCA1a and BRCA1b proteins interact with p53 *in vitro* and *in vivo*. The p53 interaction domain of BRCA1a/1b maps, *in vitro*, to the second BRCT domain (aa 1760–1863). The BRCT domain binds to the central domain of p53 which is required for sequence specific DNA binding. These results demonstrate for the first time the presence of a second p53 interaction domain in BRCA1 proteins and suggests that BRCA1a and BRCA1b proteins, like BRCA1, function as p53 co-activators. This BRCT domain also binds *in vitro* to CBP. These results suggest that one of the mechanisms by which BRCA1 proteins function is through recruitment of CBP/p300 associated HAT/FAT activity for acetylation of p53 to specific promoters resulting in transcriptional activation.

**Keywords:** BRCA1; BRCA1a; BRCA1b; p53; p21<sup>WAF1/CIP1</sup>; BRCT domain; CBP/p300

BRCA1, a familial breast and ovarian cancer susceptibility gene, is linked to 45% of the families with inherited breast cancers and about 90% of families with inherited breast and ovarian cancers (Miki *et al.*, 1994; Easton *et al.*, 1995; Ford *et al.*, 1995). BRCA1 encodes a protein of 1863 amino acids. The unique amino-terminal zinc finger domain interacts with BARD1 (Wu *et al.*, 1996), E2F transcrip-

tional factors, cyclins and cyclin-dependent kinases (Wang *et al.*, 1997). Two, tandem carboxy-terminal BRCT domains (BRCA1 C-terminal domain (Koonin *et al.*, 1996; Bork *et al.*, 1997; Callebaut and Mornon, 1997) are involved in transcription activation, growth inhibition and tumor suppression (Chapman and Verma, 1996; Monteiro *et al.*, 1996; Rao *et al.*, unpublished results; Holt *et al.*, 1996; Thompson *et al.*, 1995; Rao *et al.*, 1996; Humphrey *et al.*, 1997). The BRCT domains of BRCA1 are targets for cancer associated mutations (Couch and Weber, 1996) and are conserved evolutionary (Szabo *et al.*, 1996). Several recent reports have shown moderate homology between the BRCT domains of BRCA1 and the C-terminal region of p53-binding protein (53BP1), the yeast RAD9 protein and multiple proteins involved in cell cycle checkpoint functions responsive to DNA damage (Koonin *et al.*, 1996; Bork *et al.*, 1997; Callebaut and Mornon, 1997). Recently, we and others showed that the N- and C-terminal regions of BRCA1, BRCA1a and BRCA1b proteins activate transcription when fused to GAL4 DNA binding domain (Cui *et al.*, 1998a; Chapman and Verma, 1996; Monteiro *et al.*, 1996), and associate with RNA polymerase II holoenzyme (Scully *et al.*, 1997a) and CBP/P-300 co-activator (Cui *et al.*, 1998b), suggesting a potential role for BRCT domains in the regulation of transcription. The multiple functions of BRCA1 protein(s) include growth/tumor suppression (Holt *et al.*, 1996; Thompson *et al.*, 1995; Rao *et al.*, 1996; Humphrey *et al.*, 1997), induction of apoptosis (Shao *et al.*, 1996), cell cycle regulated expression, DNA repair and the maintenance of genomic stability (Lane *et al.*, 1995; Marquis *et al.*, 1995; Scully *et al.*, 1997b; Brugarolas and Jacks, 1997). The BRCA1 protein physically associates, both *in vitro* and *in vivo*, with p53 tumor suppressor gene and stimulates p53-dependent transcription from the p21<sup>WAF1/CIP1</sup> and bax promoters (Ouchi *et al.*, 1998; Zhang *et al.*, 1998). BRCA1 has also been shown to transactivate expression of the cyclin-dependent kinase inhibitor p21 in a p53-independent manner (Somasundaram *et al.*, 1997), suggesting that one of the mechanisms by which BRCA1 regulates cell cycle and suppresses growth is through the induction of p21 (Somasundaram *et al.*, 1997).

p53 is a tumor suppressor protein that is altered in numerous human malignancies including colon, lung, breast, ovary and several others (Hollstein *et al.*, 1991). The p53 protein has been implicated in a number of functions, including cell cycle regulation, response to DNA damage, signal transduction, cellular differentiation and activation and repression of transcription (Ko



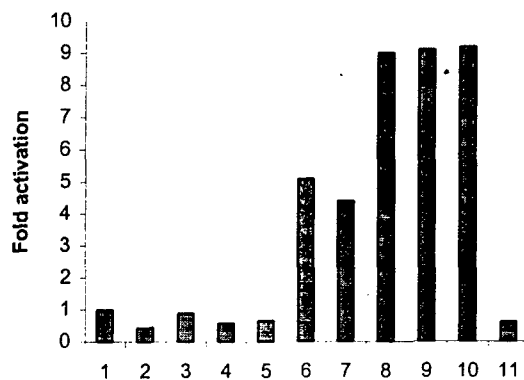
**Figure 1** Direct physical association of p53 with BRCA1a, BRCA1b and the second BRCT domain of BRCA1. *in vitro*. (a) GST, GST-BRCA1a, GST-BRCA1b and GST-BRCT domain (amino acids 1760–1863) proteins were expressed, purified on beads and incubated with *in vitro* translated <sup>35</sup>S-methionine-labeled p53 and subjected to GST-pull down assay as described

and Prives, 1996). The p53 protein contains distinct functional domains: a transcriptional activation domain at the amino terminal (residues ~1–70); a SH3-like domain (residues ~61–94); a central sequence-specific DNA binding domain (residues ~102–392); a nonspecific DNA-binding domain at the carboxy terminal region (residues ~320–393) which contains a tetramerization domain (Stürzbecher *et al.*, 1992); and a basic domain (Ko and Prives, 1996 and references therein).

The presence of a common BRCT domain between BRCA1 and p53 binding protein 53BP1 (Iwabuchi *et al.*, 1994), which is also present in splice variants BRCA1a and BRCA1b (Cui *et al.*, 1998a), led us to investigate whether BRCA1 proteins associate with p53, the universal tumor suppressor, as speculated earlier by others (Bork *et al.*, 1997; Koonin *et al.*, 1996). To determine whether intact BRCA1a/1b and a deletion mutant (containing the second BRCT domain aa 1760–1863) bind with p53, *in vitro* binding was performed with full length wild type human p53 protein. The *in vitro* translated <sup>35</sup>S-methionine-labeled p53 protein was passed through GST-BRCA1a, GST-BRCA1b, GST-BRCT domain (residues 1760–1863) and a GST control column as described previously (Wang *et al.*, 1997; Cui *et al.*, 1998b). p53 was found to bind specifically to GST-BRCA1a, GST-BRCA1b, GST-BRCT domain (residues 1760–1863) but not to GST (Figure 1a, lanes 3, 4 and 5). The region of BRCA1a/1b extending from aa residues 1760–1863, which also maps to the minimum transactivation domain, was found to be sufficient for interaction with p53. Recently, the N-terminal region of exon 11 (aa 224–500) was shown to interact with the C-terminal domain (aa 300–393) of p53 (Zhang *et al.*, 1998). Interestingly, majority of exon 11 (aa 263–1365) is lost in BRCA1a and BRCA1b (Cui *et al.*, 1998a; Rao, unpublished results) but they still bind to p53. Our results demonstrate for the first time a second p53 interaction domain in the second BRCT domain of BRCA1 protein. To map the region of p53 which binds to the BRCT domain, a series of p53 deletion mutants expressed as GST fusion proteins (generous gifts from Drs Shenk and Wiman) were incubated with *in vitro* translated BRCT domain (aa 1760–1863) protein and subjected to GST-pull down assay. The results show that BRCT domain binds to the central region but not to the C-terminal region (amino acids 319–393) (Figure 1b, top panel lanes 3 and 4, bottom panel lane 3). These results suggest that the BRCT domain of BRCA1 protein binds to the central domain of p53 similar to 53BP1, 53BP2 and SV40 large tumor antigen. This central domain is responsible for site

previously (Wang *et al.*, 1997; Cui *et al.*, 1998b). In lane 1, 1/20th of the *in vitro* translated protein used for binding was loaded directly. The second protein band seen in lane 1 represents polypeptide arising as a result of initiation at the internal methionine codon or premature termination. (b) BRCT domain (amino acids 1760–1863) binds to the central domain of p53 (amino acids 99–307). GST, GST-p53 (amino acids 1–160), GST-p53 (amino acids 160–318), GST-p53 (amino acids 319–393) and GST-p53 (amino acids 99–307) proteins were expressed, purified on beads and incubated with *in vitro* translated <sup>35</sup>S-methionine-labeled BRCT domain (amino acids 1760–1863) and subjected to GST-pull down assay as described above

specific DNA binding and contains mutations that are found commonly in tumors. Since BRCA1a, BRCA1b and the BRCT domain interact *in vitro* with p53, we investigated the effects of BRCA1a/1b and BRCT on p53 independent and p53 dependent transcription. Since both p53 and BRCA1 can transactivate the p21 promoter (El-Deiry *et al.*, 1995; Somasundaram *et al.*, 1997), we studied the effects of BRCA1a and BRCA1b on the p21 promoter. pcDNA-BRCA1a, pcDNA-BRCA1b or pcDNA-BRCT (1760–1863), both alone and in the presence of pSGp53 were co-transfected into COS-7 cells together with a natural genomic p21 promoter CAT vector (a generous gift from Dr Volgelstein) that contains the p53 response element (El-Deiry *et al.*, 1995). Neither BRCA1a, BRCA1b nor BRCT domain (residues 1760–1863) activated the p21 promoter in the absence of exogenous p53 (Figure 2), but all stimulated the p21 promoter in the presence of p53 (Figure 2). These results suggest that BRCA1a and BRCA1b proteins function as co-activators of p53 and the C-terminal second BRCT domain of BRCA1/1a/1b proteins is sufficient for binding to p53 and stimulating its target gene activation. As mentioned earlier, several groups have shown that BRCA1 regulates p21 through both p53-dependent and p53-independent mechanisms (Ouchi *et al.*, 1998; Zhang *et al.*, 1998; Somasundaram *et al.*, 1997). Since BRCA1a/1b lack the N-terminal region of exon 11 which was also shown to interact with p53, we speculate that BRCA1a/p110 and

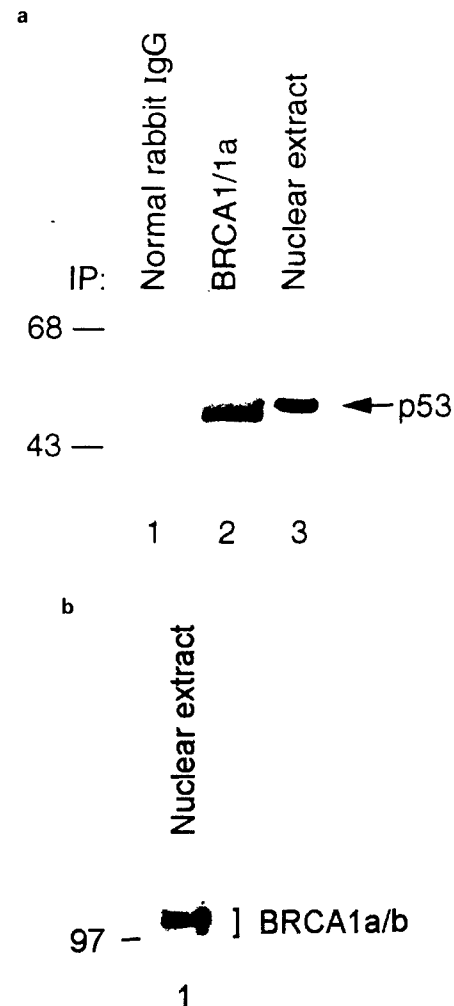


|              |   |   |   |   |   |   |   |   |   |   |   |
|--------------|---|---|---|---|---|---|---|---|---|---|---|
| PCDNA        | + | - | - | - | - | - | + | - | - | - | - |
| BRCA1a       | - | + | - | - | - | - | - | + | - | - | - |
| BRCA1b       | - | - | + | - | - | - | - | - | + | - | - |
| BRCA1a/b     | - | - | - | + | - | - | - | - | - | + | - |
| aa-1760-1863 |   |   |   |   |   |   |   |   |   |   |   |
| PSG5         | - | - | - | - | + | - | - | - | - | - | - |
| p53 WT       | - | - | - | - | - | + | + | + | + | + | + |
| mu. Rep      | - | - | - | - | - | - | - | - | - | - | + |

**Figure 2** The second BRCT domain of BRCA1/1a/1b is sufficient for p53 dependent transactivation of the p21<sup>WAF1/CIP1</sup> promoter. COS-7 cells were co-transfected with 1 µg of human p21 promoter CAT 'A' or 'E' construct (El-Deiry *et al.*, 1995) and 1 µg of expression plasmids (pcDNA-BRCA1a, pcDNA-BRCA1b, pcDNA-BRCT amino acids 1760–1863 and pSGp53 wild type) as described previously (Cui *et al.*, 1998a,b). Total DNA was kept constant at 20 µg. The CAT activity shown represents fold activity compared with pcDNA vector alone. The activity of the vector is normalized to a value of one. The experiments were repeated at least four times. Mu-Rep represents p21 promoter construct 'E' (El-Deiry *et al.*, 1995) which lacks p53 binding sites. The results of CAT assay were quantitated using a Fuji phosphorimager

BRCA1b/p100 can regulate p21 transcription only through a p53-dependent mechanism. All these results taken together suggest a role for p53 in the growth arrest, tumor suppression and apoptosis-inducing functions of BRCA1 proteins.

To study these associations in a physiologically relevant environment, cell extracts from human breast cancer cells CAL-51 were immunoprecipitated with anti-BRCA1 polyclonal antibody and as a negative control, normal rabbit IgG. Subsequent Western blot analysis with anti-p53 monoclonal antibody revealed p53 in the anti-BRCA1 immunoprecipitates (Figure 3a, lane 2), and nuclear extract (Figure 3a, lane 3), but not in the control immunoprecipitate (Figure 3a, lane 1). The anti-BRCA1 antibody C-20 detects both BRCA1a/1b proteins by immunoprecipitation and Western blot analysis (Figure 3b). All these results suggest that BRCA1a/1b proteins associate with p53 *in vivo*.

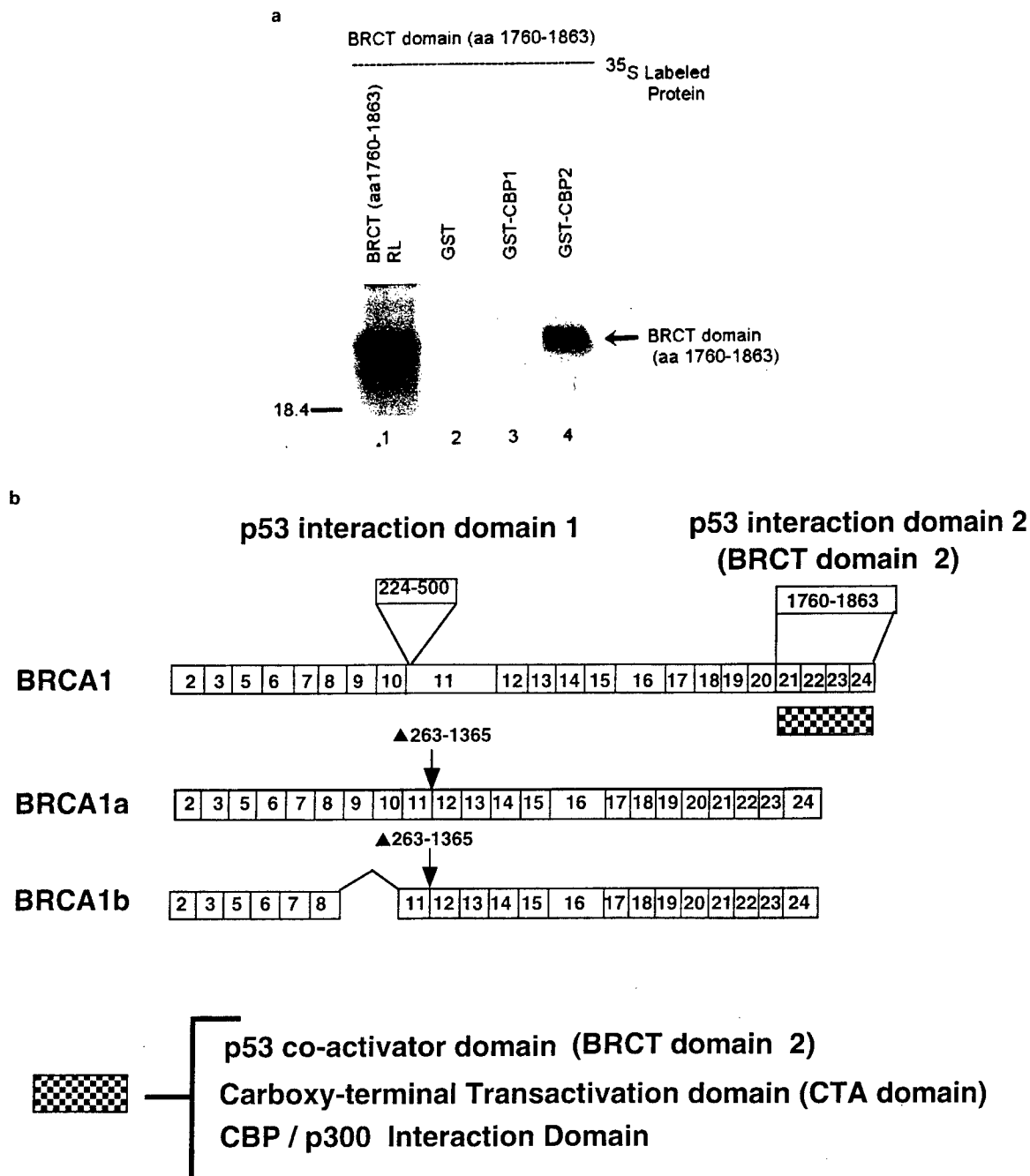


**Figure 3** *In vivo* interaction of p53 with BRCA1a/1b proteins. (a) BRCA1 proteins were immunoprecipitated by anti-BRCA1 antibody from cell extracts of human breast cancer cell line CAL51, separated on 10% SDS-PAGE analysis and Western blotted with anti-p53 monoclonal antibody. Lane 1, immunoprecipitation by normal rabbit IgG (negative control), lane 2, immunoprecipitation by anti-BRCA1 antibody, lane 3 A431 nuclear extract (positive control for p53). (b) Western blot analysis of nuclear extract from A431 cells using anti-BRCA1 antibody after immunoprecipitation with anti-BRCA1 antibody was done as described (Cui *et al.*, 1998b). The protein bands shown represent endogenous BRCA1a (p110) and BRCA1b (p100) proteins

BRCA1 associates with RNA polymerase II holoenzyme complex (Scully *et al.*, 1997a) and CBP (CREB-binding protein) is a component of the holoenzyme (Nakajima *et al.*, 1997). Previously, we have found BRCA1a/p110 and BRCA1b/p100 to interact both *in vitro* and *in vivo* with the carboxy-terminal domain of transcription factor CBP (Cui *et al.*, 1998b). Since the BRCT domain is sufficient for p53 mediated transactivation of the p21 promoter, we speculated whether the BRCT domain could interact directly with CBP which would bring the RNA polymerase II holoenzyme into play. Our results using GST-pull down assays demonstrate direct physical interaction between GST-CBP2 and *in vitro* translated BRCT domain of BRCA1

(Figure 4a). These data are consistent with the notion that BRCA1 functions as a transcriptional co-activator.

In summary, our results suggest BRCA1a and BRCA1b proteins function as co-activators of p53 tumor suppressor protein similar to BRCA1. This study demonstrates for the first time the presence of a second p53 interaction domain in the carboxy terminal BRCT domain of BRCA1 (Figure 4b), which is sufficient for activation of p53 dependent transactivation of the p21<sup>WAF1</sup> promoter. Previously, based on its homology with p53 binding protein 53BP1, it was speculated that the BRCT domain could bind p53 (Koonin *et al.*, 1996; Bork *et al.*, 1997) and our result



**Figure 4** (a) BRCT domain (aa 1760–1863) interacts *in vitro* with CBP2 (aa 1620–1877). GST, GST-CBP1 or GST-CBP2 were incubated with *in vitro* translated <sup>35</sup>S-labeled BRCT domain (aa 1760–1863) and subjected to GST-pull down assay as described previously (Cui *et al.*, 1998b). The faint band in lane 3 represents weak binding of BRCT domains to GST-CBP1. (b) Schematic representation of the p53 interaction domains of BRCA1, BRCA1a and BRCA1b proteins.

confirms this hypothesis. Since BRCA1a, BRCA1b, and BRCT domain (residues 1760–1863) proteins associate with CBP/p300 co-activator (Cui *et al.*, 1998b and this paper) and the C-terminal truncation, Y1853X, fails to bind to RNA polymerase II holoenzyme (Scully *et al.*, 1997a) nor activate transcription (Somasundaram *et al.*, 1997; Chapman and Verma, 1996; Monteiro *et al.*, 1996; Ouchi *et al.*, 1998; Zhang *et al.*, 1998), we speculate that one of the mechanisms by which BRCA1 proteins function is through recruitment of CBP/p300 associated HAT/FAT activity for acetylation of p53 to specific promoters resulting in transcriptional activation. Our results demonstrate binding of the BRCT domain of BRCA1 (residues 1760–1863) to the central region of p53 (amino acids 79–307) but not to the C-terminal region (amino acids 319–393), which was earlier shown to bind to the N-terminal region of exon 11 of BRCA1 (amino acids 224–500). This central region of p53 contains the sequence specific DNA binding domain which is required for the tumor suppressor function of p53 protein (Ko and Prives, 1996 and references therein). The majority of p53 missense mutations in tumors are present within this central region. The binding of the BRCT domain of BRCA1 proteins to p53 suggests that it may be involved in p53 mediated tumor suppression, apoptosis, and DNA repair. It is possible that the BRCT domain may be involved in determining the specificity of p53 binding to high and low affinity p53 binding sites resulting in either p53-dependent transcriptional activation or repression. It is conceivable that the BRCT domain may activate p53 by inducing a conformational change resulting in increased DNA binding and transactivation. Our findings suggest that the BRCT domain binding to p53 may be mediated by CBP/p300, since it binds to the same region on BRCA1 and this complex

may in turn be linked to RNA polymerase II holoenzyme complex. In fact, 87% of mutations that have been reported in familial breast cancer disrupt the BRCT domains by truncation of the protein (Couch and Weber, 1996), while some others disrupt the domains by missense mutations. It remains to be seen what effect these cancer-predisposing mutations have on the p53 binding and co-activator function of BRCA1 proteins. The p53 binding/co-activator property of BRCT domain can be used as an assay for detecting functionally relevant alterations in patients with BRCA1 mutations. It is possible that lack of binding of disrupted BRCA1 proteins to p53 in patients with mutations in the BRCT domain could lead to the development of breast cancer. The binding of the BRCT domain to p53 suggests that other BRCT domain containing proteins involved in cell cycle checkpoint functions responsive to DNA damage may similarly interact with p53. Lastly, although we have shown BRCA1a/1b to activate p53 dependent transcription of the p21 promoter in episome-based assays, the question whether the same holds true for endogenous p21 remains to be investigated.

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# Structure and expression of variant BRCA2a lacking the transactivation domain

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**Abstract.** BRCA1 and BRCA2 are tumor suppressor genes shown to be involved in 90% of familial breast cancers and also known to be involved in ovarian and prostate cancers. Both BRCA1 and BRCA2 gene products are regulated in a cell cycle-dependent manner and have potential transactivation function. Here, we show that BRCA2 undergoes differential splicing giving rise to a novel variant protein BRCA2a, lacking putative transcriptional activation domain. Both BRCA2a and BRCA2 are expressed at high levels in thymus and testis but moderate levels in mammary gland and prostate suggesting that BRCA2a and BRCA2 may have a role in the development and differentiation of these tissues.

## Introduction

Germ-line mutations in autosomal dominant susceptibility genes are responsible for up to 10% of all breast cancers (1,2). Mutations in breast cancer susceptibility genes, BRCA1 and BRCA2, could account for up to 90% of familial breast cancers (3-5). Recently BRCA1 and BRCA2 have also been shown to be associated with ovarian and prostate cancers. Interestingly, unlike BRCA1, BRCA2 is associated with male breast cancer (4). The BRCA2 gene is composed of 27 exons and encodes a protein of 3418 amino-acids with no significant homology to any known protein (6,7). Expression of both BRCA1 and BRCA2 was shown to be cell cycle regulated and expressed at high levels in late G1 and S-phase (8,9). Recently exon 3 of BRCA2 was shown to function as a transcriptional activation domain suggesting that BRCA2 may have a functional role in the regulation of transcription (10). Similar transcriptional activation function was also shown to be present in BRCA1 (11-13). Previously we have shown that BRCA1 is trans-ported into the nucleus in the

absence of serum and interacts with transcriptional factor E2F, cyclins and cdks suggesting a role for BRCA1 in cell cycle regulation (14). BRCA2 and BRCA1 proteins were shown to interact with Rad 51 suggesting that they may play a role in DNA repair (15-18).

Recently we have shown that both BRCA1 and BRCA2 interact with CBP *in vivo* and *in vitro* suggesting that both these proteins may regulate transcription through CBP (19,20). Because of coordinated expression of BRCA1 and BRCA2 genes and association of these genes in the common breast cancer phenotype, it appears that BRCA1 and BRCA2 may function in a similar pathway. Recently, we have shown that BRCA1 induces apoptosis suggesting a novel function in the regulation of apoptosis of cells (21,22). It remains to be seen whether BRCA2 plays a similar role in apoptosis. Both BRCA1 and BRCA2 proteins may have multicellular functions such as transcriptional activation, DNA repair and regulation of apoptosis.

In this study, we have cloned alternatively spliced BRCA2 cDNA and characterized them by nucleotide sequence analysis. We demonstrate that this differentially spliced BRCA2a transcript has lost transcriptional activation domain as a result of alternative splicing giving rise to BRCA2a with potential dominant negative pathophysiology. Interestingly, BRCA1 was also shown to encode multiple products as a result of alternative splicing (13,14,21,23-25).

## Materials and methods

**Molecular cloning of BRCA2 and BRCA2a cDNAs.** cDNAs were obtained by the reverse transcription of total RNA from BT-474 cells using cDNA kit (Takara). BRCA2 cDNAs were amplified by PCR using appropriate 5' and 3' primers and cloned into a pcDNA3 vector. These cDNAs were characterized by restriction mapping and nucleotide sequence analysis.

**RNase protection assay.** RNase protection assay was carried using Ribonuclease Protection assay kit (Ambion Inc., Austin TX) as described by the manufacturer. Briefly, the templates were subcloned, linearized and transcribed in 20  $\mu$ l of *in vitro* transcription mixture containing 5  $\mu$ l of  $\alpha$ -<sup>32</sup>P-rUTP to obtain radiolabelled probes. These radiolabelled RNA probes were purified by gel electrophoresis. Approximately 5x10<sup>5</sup> cpm of the probe was mixed with 20  $\mu$ g of human breast, prostate, testis and thymus RNA (Clontect, Palo Alto, CA) and the

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**Key words:** BRCA2a, BRCA2, alternative splicing, transcriptional activation, tumor suppressor, HAT activity

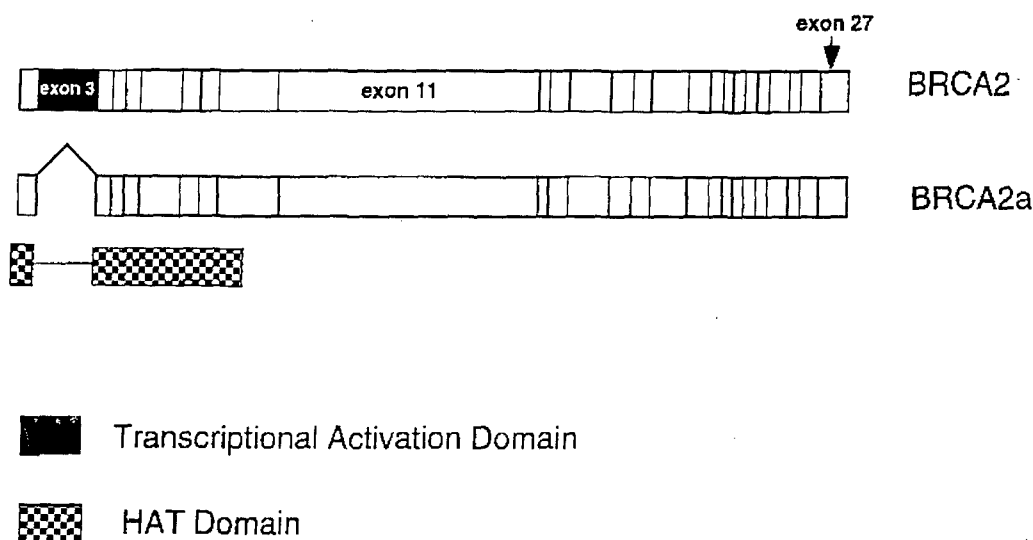


Figure 1. Deletion of BRCA2 transcriptional activation domain in BRCA2a as a result of alternative splicing. Figure is not drawn to scale.

volume of the reaction mixture was adjusted to 30  $\mu$ l by 1X hybridization buffer. The hybridization mixture was heated to 95°C for 5 min and then incubated at 45°C overnight. RNase digestion was performed for 1 h at 37°C and the reaction was stopped by the addition of RNase inactivation buffer (kit). The protected fragments were extracted by centrifugation. The pellets were suspended in gel loading buffer, heated to 90°C for 3 min. The reaction products were analyzed by polyacrylamide gel electrophoresis using 5% polyacrylamide/8M urea gels. The gel was dried and subjected to autoradiography.

### Results and Discussion

In order to understand the function of BRCA2, we have cloned several cDNAs by RT PCR and characterized these cDNAs by nucleotide sequence and restriction map analysis. Our results demonstrate that one of the cDNAs (BRCA2a) showed alternative splicing resulting in the deletion of exon 3 (Fig. 1). Previously this exon was shown to contain a potential transcriptional activation domain, which suggested that BRCA2 may function as a transcriptional factor (10). Similar potential for transcriptional activation was attributed to BRCA1 proteins (11-13). Since BRCA2a has lost transcriptional activation domain, it might compete with native BRCA2 in terms of DNA binding or interaction with other transcriptional factors resulting in dominant negative effect on transcription activation function of BRCA2. Such dominant negative variants are also seen in other transcriptional activators (26). Therefore, BRCA2a may represent a potential dominant negative variant which may regulate the putative transcriptional activation properties of BRCA2 proteins. Alternatively, BRCA2a may have other functions which do not need transactivation function.

We performed RNase protection analysis to study the expression of BRCA2 and BRCA2a in different types of tissues. For this, we have used the 459 nucleotide probe (Fig. 2a). The predicted 313 nucleotide fragment (corresponding to BRCA2a) and the 255 and 58 nucleotide

fragments corresponding to BRCA2 were observed in thymus and testis (Fig. 2, lanes 3 and 4). However, moderate to low level of expression was observed in the case of mammary gland and prostate (Fig. 2, lanes 5 and 6). It appears both BRCA2 and BRCA2a are expressed at similar levels in the tissues tested suggesting both forms of BRCA2 may have a functional role in cell growth and differentiation of testis, mammary gland, prostate and thymus.

In summary, we have presented the results supporting that BRCA2 is alternatively spliced, giving rise to a variant BRCA2a protein which lacks transactivation domain. To our knowledge this is the first report demonstrating the presence of variant BRCA2 protein. Since BRCA2a variant lacks transcriptional activation domain, it can potentially interfere with transcriptional activation properties of BRCA2 by competing with BRCA2 for protein-protein interactions and/or DNA binding. Such variant proteins were also seen in the case of other transcriptional factors. It is possible that BRCA2a may regulate the functional properties of BRCA2. Therefore, it becomes important to study the patient DNA samples for mutations outside the coding region (introns, promoters etc.) as they may alter differential splicing pattern of BRCA2 leading to overexpression of BRCA2a. This overexpression of BRCA2a may interfere with normal BRCA2 function and result in cellular transformation. In support of this hypothesis, large deletions that disrupt exon 3 of BRCA2 were observed in patients of breast and ovarian cancers (27).

Our recent results have shown that both BRCA2 and BRCA2a have histone acetyltransferase activity (HAT) (20). These results suggest that domains responsible for HAT activity and the transcriptional activation function of BRCA2 do not overlap (Fig. 1). Since BRCA2 and BRCA2a are HAT proteins, it is possible both BRCA2 and BRCA2a function as transcriptional co-factors. BRCA1 was shown to function as a transcriptional co-factor of p53 (28,29). Recently, we have observed that BRCA2 binds to CBP and function as transcriptional co-factors of p53 (Siddique and Reddy, unpublished results). It is possible that some transcriptional

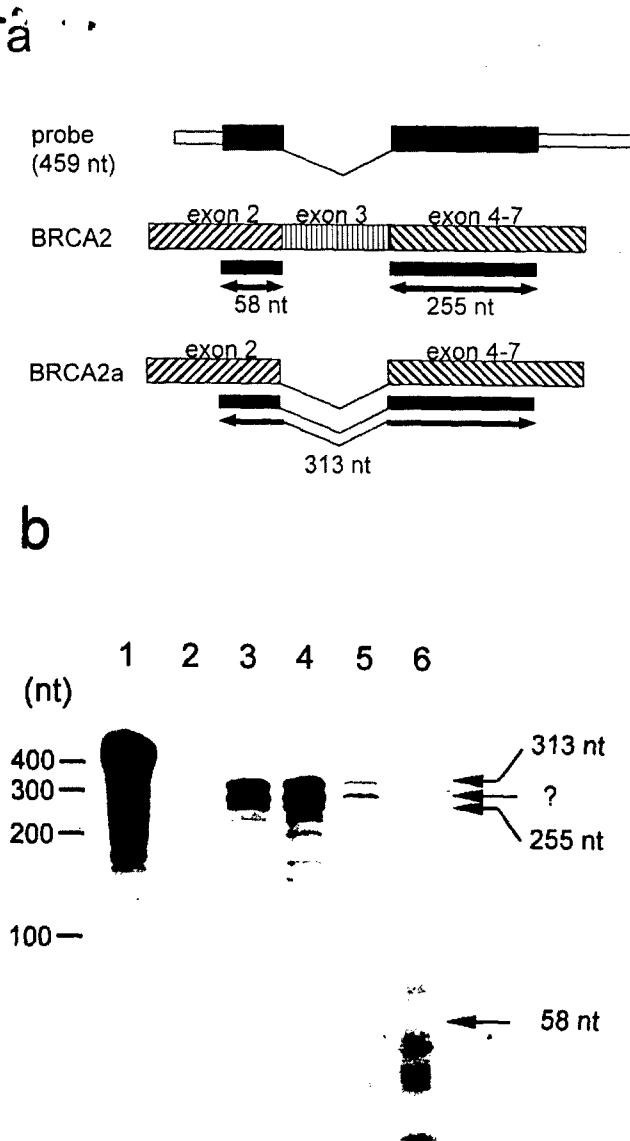


Figure 2. a) Schematic representation of antisense RNA probe used for RNase protection assay. The expected protected fragments from BRCA2 and BRCA2a transcripts are shown. b) Analysis of the expression of BRCA2a in different human tissues by RNase protection assay. This assay was carried out on 20 µg of total RNA using Ambion RPA kit. Lane 1, probe; Lane 2, tRNA; Lane 3, thymus; Lane 4, testis; Lane 5 mammary gland; Lane 6, prostate. Lanes 1-4 are the result of short exposure (1 day) and lanes 5 and 6 are of longer exposure (3 days). Protected 313 nucleotides (corresponding to the alternatively spliced BRCA2a) and 255 and 58 nucleotide fragments (corresponding to the BRCA2) are shown by arrows.

activators may use BRCA2 and BRCA2a as a transcriptional co-factors and utilize their HAT activity for the activation of gene expression. It is also possible that other transcriptional activators may use BRCA2 but not BRCA2a as a transcriptional co-factor because of the absence of the transcriptional activation domain in BRCA2a. Recent studies revealed that BRCA2 interacts with DNA repair protein Rad 51 (15-17). Earlier, we suggested that BRCA2-Rad 51 complex may disrupt nucleosomal structure using HAT activity of BRCA2 and thereby recognize damaged DNA prior to DNA repair process (20). For such a mechanism, there may not be a need for transactivation function. Therefore, BRCA2 and BRCA2a may have distinct

functions. Rad 51-BRCA2a (which lacks transactivation domain) complex may play a role in DNA repair whereas BRCA2 may play a role in the regulation of transcription. It remains to be seen whether both isoforms of BRCA2 play a role in this DNA repair phenomenon. Alternatively, BRCA2 proteins may participate in DNA repair and tumor suppression through BRCA2 target genes.

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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant DAMD17-94-J-4280. Request the limited distribution statement for Accession Document Numbers ADB225278, ADB234473, ADB249639, and ADB259022 be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by email at judy.pawlus@amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART  
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